EFFECTS OF UNPREDICTABLE CHRONIC MILD STRESS ON ADOLESCENT RATS

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Bachelor of Arts in Psychology, Elizabethtown College, 2004

Submitted to the Graduate Faculty of

University of Pittsburgh School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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EFFECTS OF UNPREDICTABLE CHRONIC MILD STRESS ON ADOLESCENT RATS

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University of Pittsburgh, 2014

Adolescence is a period of many behavioral, physiological, and neural changes, and has been considered to be a period of vulnerability to neuropsychiatric disorders. Individual differences in early life experiences or response strategies may be able to identify those most at risk and, conversely, most resistant to the development of neuropsychiatric disorders such as depression, anxiety, and addiction. Although much is known about the effects of early life stress on adult outcomes, a critical missing piece of information in the field is what the effects of chronic stress during adolescence are in adolescence. Unpredictable chronic mild stress (UCMS) is a widely-used model for inducing depressive- and anxiety-like behaviors in adult rodents that has also been shown to have differential outcome based on individual differences in novelty-seeking. This thesis investigates the immediate behavioral, neuroendocrine, and neurobiological effects of UCMS during adolescence, to fill a critical void in the current literature on adolescent stress exposure. It also examines whether individual differences in adolescents are associated with differential consequences of unpredictable stress during adolescence. I show that UCMS during adolescence does not induce a depressive- or anxiety-like phenotype, but instead evokes hyperactivity and decreased anxiety, and that peri-weaning experience can modulate the effect of UCMS. Further, we show that a response strategy strongly predictive of susceptibility/resistance to depression in adult rats — locomotor activity in a novel environment — is not yet a stable

response attribute in adolescent rats and consequently not a valuable predictive tool. Taken together, our work suggests that adolescents may be resistant to depressive- and anxiety-like consequences induced by chronic mild stress, and that stronger stressors may be required to induce a depressive-like phenotype in adolescence. In light of some evidence showing that UCMS during adolescence produces a depressive phenotype in adulthood, our work suggests that post-adolescent processes play a role in the development of the adult outcome.

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ACKNOWLEDGEMENTS

I could not have done this alone. My time in the Department of Neurobiology, Center for Neuroscience at the University of Pittsburgh, and the Center for the Neural Basis of Cognition, has been full of tremendous support, encouragement, camaraderie, and incredible science. First and foremost, I thank my advisor, Edda (Floh) Thiels, for having me in her lab. She has devoted much time, energy, and care into my graduate training. Floh's enthusiasm for telling a story with scientific results, commitment to public/educational outreach, and dedication to her family is quite inspiring. I have learned so much from her as a woman in science, and have always felt her support of my own scientific and personal growth. She has allowed me to experience the highs and the lows of what it means to be a successful scientist, and I am no doubt better for it. I thank her for allowing me to conduct my dissertation work on a topic that was not related to what the lab typically studies, and for her continued support of my project, despite the many bumps along the road.

I am grateful to the members of my committee Dr. Alan Sved (my chair), Dr. Eric Donny, Dr. Linda Rinaman, Dr. Etienne Sibille, and Dr. Don DeFranco for the guidance and support they have provided to help shape my dissertation project. Often their insights have served to strengthen my experimental work, particularly when they would remind me not to be "too nice" to my subjects. I would especially like to thank Alan and Eric for allowing me to conduct the majority of my dissertation studies in their labs, particularly when they could be

time- and labor-intensive. Thank you to Alan for spending time collecting the tissue samples as well. I would like to thank Don for suffering through rather lengthy discussions of behavioral findings, to finally be able to offer critical advice on glucocorticoid receptors and the underlying signaling that may be at play. I thank Linda for always providing a fresh perspective in the face of negative findings, encouraging me to think deeper about what they mean; and I thank her for making her lab and personnel available for me as needed. I thank Eric for his critical assessment of the data, and for reminding me to consider what it is I am actually measuring. Thank you to Etienne for providing expertise on the intricacies of my stress model, and for reminding me of the underlying broader discussions. I would also like to thank my outside examiner, Dr. Seema Bhatnagar, for taking the time to participate in my dissertation defense as an external examiner. I am honored to have someone so knowledgeable in the field of chronic stress as a member of my committee.

I thank all the past and current members of the Thiels lab for years of support. In particular, I thank Jocelyn Mauna for her friendship, encouragement, and for teaching me everything I know about Western blotting. I thank her for the immense technical support in completing my thesis as well. I am grateful to the Thiels lab and CNUP summer students who aided in the enormous task of scoring behavioral data, particularly Nikki Marinsek, Jocelyn Lu, and Carly O'Connor. I thank all the past and current members of the Sved/Donny lab, for providing support in maintaining my rodent stress studies. In particular, I thank Maysa Gharib, Emily Pitzer and Josh Alberts, for their assistance in animal care and supplies; and thank you to Barbara Cohen and Cliff Greenfield Jr. for caring for my rats and accommodating for my unique schedule of stressors. I am extremely grateful to Kristen Stedenfeld, a former Sved-lab CNUP graduate student, for helping me to start my UCMS work, for her willingness to share equipment

and even some of her own data in the beginning of my studies, and for her continued encouraging words and friendship. Finally, I thank Eloise Peet, for her friendship and immense technical support in getting my behavioral experiments started, and for always helping me to trouble-shoot.

I would like to thank the CNUP students and faculty for fostering a positive and collaborative graduate school environment. I am so grateful for the opportunities and interactions that I have experienced here. I would like to thank Dr. Charles Bradberry for providing me the opportunity to receive a training fellowship, and for his genuine conversations about my thesis work. I also thank the CNUP administrative staff, especially Patti Argenzio, Joan Blaney, Jessica Dawson, and Marlene Nieri. No matter which end of campus I was on, I always had friendly encouragement and all the support that I needed. I thank CNBC administrator Lisa Bopp for all of her assistance as well. Thank you also to Hunter Simpson, for always providing IT support.

I am grateful to all my friends and family who have continued to support and motivate me throughout my graduate career. I thank my fellow CNUP classmates, Andrea Lordan, Dr. Sonya Giridhar, Dr. Caitlin Moyer, Dr. Mike Bienkowski, Dr. Amanda Kinnischtzke, Dr. Chris Divito, and Jesse Wood. From lunches after our first-year classes to weddings and new careers, we have formed incredible bonds. I am particularly grateful to Andrea, Caitlin, Sonya, and Amanda for their long-lasting friendships; and to Mike for his words of scientific wisdom, and for introducing me to my husband! I thank my wonderful husband Drew for all the love, encouragement, and understanding, as I come to the final push in my graduate work. I am truly a better person from having found him. Finally, I thank my amazing family, without whom I would not be the person I am today. I thank my parents Scott and Peggy Zimmerman, for providing me with the spark of interest in science when I was very young, for encouraging my

creativity and academic success, and for continuing to foster my personal and professional growth. Thank you to my siblings. To my twin sister Kelly – I think that my strong drive for collaboration has stemmed from the fact that I have had a life-long partner; I hope that Autumn is ok with her auntie telling her all about the brain. Thank you to my brothers Ryan and Brendan – I believe collectively we have improved each other's performance in school and in life. Thank you to my "other sister" Danielle Alexander, for the long-time friendship; and for the willingness to discuss the ups and downs of Neuroscience research. I thank my grandmother, JoAn Zimmerman, for being a cornerstone of the Zimmerman family, and for finally being ok with me "going all the way to Pittsburgh" for graduate school. Thank you to the new families I've adopted along the way for their continued support as well: the Reider family, and the Kirschmann family.

LIST OF ABBREVIATIONS

ADHD – Attention-deficit hyperactivity disorder

BDNF – Brain-derived neurotrophic factor

Con – Controls

CREB – Cyclic-adenosine monophosphate response element-binding protein

DEX – Dexamethasone

HPA – Hypothalamic-pituitary-adrenal

ELISA – Enzyme-linked immunosorbancy assay

ERK – Extracellular signal-regulated kinase

FST – Forced swim test

In-house – Group of rats born in-house

LDH – Lactate dehydrogenase

NeuN – Neuronal-specific nuclear protein

p – Postnatal day

pCREB – Phosphorylated CREB

pERK – Phosphorylated ERK

Ship-with-dam – Group of rats shipped with a foster dam; weaned a few days later in-house

UCMS – Unpredictable chronic mild stress

Wean-and-ship – Group of rats weaned and shipped at the same time

1.0 INTRODUCTION

Stress exposure at various times throughout life (e.g., prenatal, early-life, adulthood) can precipitate numerous neuropsychiatric illnesses, particularly depression and anxiety-related disorders. One point in life that is garnering more attention in neuropsychiatric research is that of adolescence. Adolescence is a time of great transition, and is often considered to be a period of vulnerability; numerous neuropsychiatric disorders begin to arise in adolescence, or as a direct result of adolescent experiences (Spear, 2000). The period of adolescence could alternatively be protective, because some developing systems may be better suited to adapt to perturbations, like stress.

As I will review in the following sections, understanding the effects of stress exposure during adolescence *on adolescents* is an important, yet not well-studied, topic in the field. Individual differences may help identify those most at risk – or those resistant – to the effects of stress, but standard variables used in identifying vulnerability in adults may not be appropriate for this younger age group. Early-life environmental conditions, on the other hand, may serve as an organizing variable for predicting the effects of chronic stress in adolescents, and *may even determine* behavioral set-points prior to any stress exposure. This thesis investigates the behavioral, neuroendocrine, and neurobiological effects of unpredictable chronic mild stress during adolescence, in an attempt to fill a critical void in the current literature on adolescent

stress exposure. It also examines whether individual differences in adolescents are associated with differential consequences of unpredictable stress during adolescence.

1.1 STRESS AND THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Stress is broadly defined as any real or perceived threat to homeostasis or subjective well-being, and includes physical as well as psychological insults (Herman, 2013; McEwen, 2007; Ulrich-Lai & Herman, 2009). How an individual reacts to conditions of stress can determine his/her ultimate survival or demise. Immediate removal of oneself from a stressful situation is adaptive; however, prolonged activation of stress response systems after the immediate threat is gone can be detrimental. Response to stress involves two components: an early autonomic response, mediated via activation of the sympathetic nervous system, i.e., the body's "fight-or-flight" response, and an endocrine response, mediated via activation of the hypothalamic-pituitary-adrenal (HPA) axis (Herman, 2013; Lupien et al., 2009; McEwen, 2007; Sapolsky et al., 2000). Our focus for this work is the HPA axis stress response.

When a threat is detected, a series of hormones are induced to ultimately cause the release of glucocorticoids (cortisol in humans, corticosterone in rodents). First, neurons in the medial parvocellular region of the paraventricular nucleus of the hypothalamus release corticotrophin-releasing factor, which triggers the release of adrenocorticotropic hormone from the pituitary gland; the adrenocorticotropic hormone then enters the blood stream and stimulates production of glucocorticoids by the adrenal cortex. The secreted glucocorticoids act on numerous tissues to mobilize energy stores, and aid in sympathetic functions, such as vasoconstriction (Herman, 2013; Sapolsky et al., 2000). When glucocorticoids are released in

response to stimulation of the HPA axis, they provide negative feedback to the system by binding to receptors in the hypothalamus, hippocampus, and prefrontal cortex, and promoting inhibition of further glucocorticoid release (Herman, 2013). Glucocorticoids bind to two main types of receptors: glucocorticoid and mineralocorticoid; once bound, these receptors can act as transcription factors and regulate gene expression (De Kloet et al., 1998; Lu et al., 2006). Both types of corticosteroid receptors are found throughout the brain; glucocorticoid receptors are expressed ubiquitously in neurons and glia, with highest concentrations in the paraventricular nucleus, the hippocampus, the amygdala, and the prefrontal cortex (Ahima et al., 1991; De Kloet et al., 1998; De Kloet, 2014). The mineralocorticoid receptor has a higher affinity for glucocorticoids, and is thought to regulate basal functions such as circadian variation under endogenous glucocorticoid levels. In contrast, the glucocorticoid receptor has a lower affinity for and is bound only at high levels of glucocorticoids, and thus is activated after stress (Herman, 2013; Lu et al., 2006; Ulrich-Lai & Herman, 2009). Glucocorticoid receptor transcriptional regulation has recently been shown to be altered by phosphorylation of the receptor at multiple sites (Adzic et al., 2009; Chen et al., 2008; Galliher-Beckley & Cidlowski, 2009; Weigel & Moore, 2007). Two sites of particular interest on the human glucocorticoid receptor are serine-211 and serine-226: increased transcriptional activation, and increased expression of glucocorticoid receptor-responsive genes have been shown when relative levels of phosphorylation at serine-211 exceed relative levels of phosphorylation at serine-226 (Chen et al., 2008).

Activation of the HPA axis is necessary for survival; if an individual is confronted with an aggressor, activation of the stress response enables escape from the imminent harm. However, prolonged activation from periods of chronic stress or improper termination of the evoked

response can have long-term negative consequences. For example, prolonged exposure to glucocorticoids can cause hippocampal atrophy and inhibit neurogenesis (Herman et al., 1995; Isgor et al., 2004). As is the case with many phenomena, there appears to be an "inverted U-shaped" relationship between behavioral function and physiological response: very high levels of glucocorticoids are detrimental (as discussed above), very low levels result in sub-optimal response patterns, but mild-moderate levels of glucocorticoids can be beneficial. For example, it has been shown that corticosterone administration immediately after a spatial learning task improves subsequent spatial memory in rodents (Sandi et al., 1997). Additionally, research has shown that humans actually perform better under a bit of stress [reviewed in: (Lupien & McEwen, 1997)]. Hence, a little bit of stress could be helpful.

1.2 STRESS-RELATED NEUROPSYCHIATRIC DISORDERS

1.2.1 Consequences of early-life stress

Exposure to stress early in life can have drastic influence on behavioral and endocrine stress response (Levine, 2005). Human studies have shown that early life stress is a major risk factor for numerous neuropsychiatric disorders, including depression, anxiety, and attention-deficit/hyperactivity disorder later in life (Heim & Nemeroff, 2001). In rodents, long periods of deprivation of maternal care have been shown to cause depressive- and anxiety- like behaviors and over-active HPA axis function in adolescence and adulthood (Anisman et al., 1998; Francis et al., 1999; Ladd et al., 2004; Workel et al., 2001). Also, environmental perturbations in pre-weanling rats have been shown to reduce glucocorticoid receptor gene expression in the apex of

the HPA axis, the paraventricular nucleus of the hypothalamus, while also increasing basal corticosterone levels, in adulthood (Avishai-Eliner et al., 2001; Gilles et al., 1996). Two stress-related neuropsychiatric disorders of relevance to this work, and the known underlying neurobiology, are discussed below.

1.2.2 Major Depressive Disorder

Major Depressive Disorder affects ~16% of the US adult population at least once in their lifetime (American Psychiatric Association, 2013; Kessler et al., 2008; Nestler et al., 2002; Schmidt & Duman, 2007). In addition to the disease's effect on the individual, there are tremendous secondary costs to society, including an economic burden of over 110 billion dollars annually in the US alone (Mrazek et al., 2014). Major Depression is diagnosed based on criteria in the Diagnostic and Statistical Manual (American Psychiatric Association, 2013; Krishnan & Nestler, 2008; Nestler et al., 2002). An individual is diagnosed with Major Depression when he/she presents a combination of symptoms over a two-week period that is disruptive to social and occupational function. These symptoms include: depressed mood, irritability, sleep changes, weight changes, feelings of hopelessness, worthlessness, and guilt, suicidal ideation, or decreased interest in pleasurable stimuli (anhedonia) (American Psychiatric Association, 2013; Krishnan & Nestler, 2008; Nestler et al., 2002). Depressed patients can often have co-morbid anxiety disorders as well (American Psychiatric Association, 2013; Beesdo et al., 2010; Kessler et al., 2008; Wittchen et al., 2003).

Research utilizing animal models and human genotyping and postmortem studies on the neurobiology of depression has resulted in hypotheses relating to stress/HPA axis alterations (Baune et al., 2009a; Gourley et al., 2008b; Kunugi et al., 2010), neurogenesis and neurotrophic

factors such as brain-derived neurotrophic factor (BDNF) (Duman & Monteggia, 2006; Eisch et al., 2003; Nestler et al., 2002), cytokines such as interleukin-1β (Anisman et al., 2008; Bilbo, 2009; Goshen & Yirmiya, 2009; Grippo et al., 2005a; Koo & Duman, 2009), and specific signaling pathways such as the cyclic adenosine monophosphate (cAMP) cascade (Liu et al., 2011; Nestler et al., 2002).

Though there is a consensus that there is HPA axis dysregulation in depressed patients, the direction of dysregulation is debatable. Some studies report elevated basal cortisol in depressed patients, but others see no change [reviewed in: (Sickmann et al., 2014)]. When depression is further characterized into subtypes based on symptoms, persons with atypical depression (marked by increased sleep and eating) exhibit lower cortisol levels (Brouwer et al., 2005). Patients with depression demonstrate blunted stress reactivity and impaired HPA axis negative feedback (Burke et al., 2005; Heim et al., 2008). Stress-induced depressive-like behaviors in rodents were also shown to correlate with increased basal corticosterone levels in the blood, and impaired HPA axis negative feedback (Grippo et al., 2005a; Helmreich et al., 2008), Thus, although the nature of the HPA axis dysregulation may have variations based on symptomology, it is clear that stress plays a role in depression.

Additional brain areas believed to be important in depression include limbic and reward-related regions such as the hippocampus, the prefrontal cortex, the amygdala, and the nucleus accumbens (Duman & Monteggia, 2006; Nestler et al., 2002). Often, the impact of stress is brain region-specific. For example, chronic stress was shown to decrease hippocampal levels of BDNF in adult rodents, and decreased hippocampal BDNF levels also were observed in depressed humans (Duman & Monteggia, 2006; Dwivedi et al., 2001; Nair et al., 2007). On the other hand, antidepressant treatment was shown to increase hippocampal BDNF levels, as well as increase

phosphorylated levels of cyclic-AMP response element-binding protein (CREB) in the prefrontal cortex (Duman & Monteggia, 2006; Nestler et al., 2002; Saarelainen et al., 2003). Paradigms designed to induced depressive-like symptoms, such as anhedonia and despair, in adult rodents have been shown to increase glucocorticoid receptor protein levels in the prefrontal cortex but reduce glucocorticoid receptor protein and mRNA levels in the hippocampus (Adzic et al., 2009). The BDNF gene is a known target of the glucocorticoid receptor (Adzic et al., 2009), which suggests a possible link between the two molecules in depression. Additionally, a main target of BDNF, extracellular signal-regulated kinase (ERK) was found to be decreased in the hippocampus of depressed patients (Dwivedi et al., 2001). Stress-induced depressive-like behaviors in rodents were shown to correlate with increased serum pro-inflammatory cytokine (e.g., interleukin-1β) levels (Grippo et al., 2005a; Stedenfeld, 2011), and decreased basal ERK phosphorylation in the hippocampus (Jeon et al., 2012; Qi et al., 2008; 2006; 2009). Adding support to the idea that increased levels of these stress/motivation-associated proteins in the nucleus accumbens are related to depression, it was shown that increased levels of BDNF in the accumbens are pro-depressive, and over-expression of a non-functioning BDNF receptor in the accumbens reverses these effects (Eisch et al., 2003). Finally, CREB is on one hand a key target of ERK, and on the other hand an upstream regulator of BDNF (Nair et al., 2007). Data indicate that CREB expression also may be altered in specific brain regions in depression (Muschamp et al., 2011; Nestler et al., 2002; Wallace et al., 2009). It is plausible that the ERK/CREB/BDNF signaling cascade could be impacted as a whole in depression, in a regionally-specific manner, and that these alterations could be influenced by changes in the glucocorticoid receptor and cytokines as well.

Changes in mood concerns are also frequently reported in adolescent humans (Angold & Worthman, 1993; Oldehinkel & Bouma, 2011). In fact, the Centers for Disease Control and Prevention list suicide as the third leading cause of death among people 10-24 years old (Centers for Disease Control and Prevention, 2010). Depressed children and adolescents have similar symptoms as depressed adults, but the underlying neurobiological changes and treatment responses are often different (Fleming & Offord, 1990). For example, depressed youth do not show evidence of the hypercortisolemia that is often reported in depressed adults, and they fail to respond to tricyclic antidepressants (Birmaher et al., 1996; Hazell et al., 1995; Kaufman et al., 2001). As the limbic circuitry implicated in depression is maturing throughout adolescence, and it is rich in glucocorticoid receptors, it could be that the adolescent brain is more vulnerable to stressors. More work is needed to understand the differences between adolescent and adult depression.

1.2.3 Attention-Deficit Hyperactivity Disorder

Attention-deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder, characterized by three main symptoms: impulsiveness, inattention, and hyperactivity (American Psychiatric Association, 2013; Andersen & Teicher, 2000; Johansen et al., 2002; King et al., 1998; Solanto, 2002). Prospective studies estimated that about 50% of children diagnosed with ADHD will continue to have symptoms into adulthood (del Campo et al., 2011). Evidence linking stress to ADHD comes from clinical work. A variety of studies have provided evidence for higher incidence of ADHD in children exposed to maltreatment early in life (Cohen et al., 2002; Famularo et al., 1992; McLeer et al., 1994). There is also a growing body of evidence for dysfunctional HPA axis function in ADHD subjects. For example, subjects who appeared to

have a persistence of ADHD symptoms over a one-year study period exhibited reduced cortisol levels in response to an acute stress, relative to subjects that did not have persistent symptoms over the one-year period (King et al., 1998). Additionally, ADHD youth had a loss of normal diurnal cortisol rhythms, which was worse in the individuals with the highest amount of hyperactivity (Kaneko et al., 1993).

The underlying pathophysiology in ADHD is thought to involve dysregulation of the dopaminergic system (Andersen & Teicher, 2000; del Campo et al., 2011; Johansen et al., 2002; Solanto, 2002). Symptoms of ADHD are usually manifested prior to puberty; in fact, diagnosis must occur by age 7 (Andersen & Teicher, 2000; Johansen et al., 2002). The timing of the onset of ADHD symptoms overlaps with a period of extensive overproduction of dopamine receptors (Andersen et al., 1997; 2000; 2002). Some molecular imaging studies have shown increased density of the dopamine transporter in ADHD patients, while others have reported decreased transporter density (Jucaite et al., 2005; Krause et al., 2000; Spencer et al., 2005). Hyperactivity in ADHD may be due to excess dopaminergic activity in the striatum. Dopaminergic increases in the striatum have been linked to hyperactivity and stereotypic behaviors in rodents (Giros et al., 1996; Wolf et al., 1996), and adolescents with ADHD have been shown to have increased striatal activity in some positron-emission tomography experiments (Ernst et al., 1999). Thus, there appears to be a link between hyperactivity and increased dopaminergic activity in ADHD.

1.3 ADOLESCENCE

Adolescence is an important period of transition from childhood into adulthood (Spear, 2000). It is a time of growing responsibility, and many social, emotional, and cognitive behaviors are established during adolescence (Doremus-Fitzwater et al., 2010; Sisk & Foster, 2004; Sisk & Zehr, 2005; Spear, 2000). Because of its transitional nature, adolescence does not have clear markers of its beginning and end, though it does include the more concretely-definable period of puberty, i.e., sexual maturation (McCormick & Mathews, 2007, 2010; Sisk & Foster, 2004; Sisk & Zehr, 2005; Spear, 2000). Characteristic behaviors of adolescence that are seen across species, including risk-taking, reward-seeking, and social interaction, may have adaptive values and can often be attributed to maturational changes occurring in the brain. Substantial remodeling of many regions, including the prefrontal cortex, the hippocampus, the amygdala, and the nucleus accumbens results from changes in dendritic spine density, synapse formation/elimination, increases/decreases in receptors, increases/decreases in neurons and glia, and shifts in projections (Casey et al., 2010; Lupien et al., 2009; McCormick & Mathews, 2007; Rubinow & Juraska, 2009; Spear, 2000).

The HPA axis has a unique profile of function during this period of development as well (Romeo et al., 2006; Spear, 2000). In rodents, the first two weeks of life are characterized by very low levels of glucocorticoids, termed the stress-hyporesponsive period, where stressors fail to evoke much of an increase in corticosterone levels (Levine, 2001; Vazquez, 1998). Throughout the course of adolescence, neuroendocrine responses to stress are distinctly different from what is seen earlier in childhood, or later in adulthood: prepubertal rats exhibit a delayed rise in, and a more prolonged release of, corticosterone in response to an acute stressor, because of incomplete maturation of negative feedback systems (Romeo et al., 2006). Additionally,

repeated exposure to the same stressor does not induce habituation of the HPA axis response in adolescents like it does in adults [reviewed in: (Lupien et al., 2009; McCormick & Mathews, 2007; Romeo, 2010; Spear, 2000; Vazquez, 1998)]. Clinical work also demonstrates that postpubertal adolescents exhibit HPA axis hyper-reactivity in response to psychosocial stressors (Gunnar et al., 2009; Hankin et al., 2010; Harkness et al., 2011).

All of these neural structures and systems that are undergoing much change during adolescence may be particularly vulnerable to physical and psychological insults such as stress (Doremus-Fitzwater et al., 2010; Heim & Nemeroff, 2001; Lupien et al., 2009; McCormick & Mathews, 2007, 2010; Vazquez, 1998). Interference with the normal circuit development/system functioning could cause long-lasting changes and render an individual more susceptible to neuropsychiatric disorders, such as schizophrenia, depression, and drug addiction (Heim & Nemeroff, 2001; McCormick & Mathews, 2007; McCormick et al., 2010). However, if stress exposure is mild and the developing systems are able to compensate for perturbed functioning, adolescents may benefit from brief stress exposure, akin to a priming effect. An example can be drawn from the field of immunology: a vaccination utilizes a small amount of something harmful (e.g., a virus), in order to provide protection from future insults (e.g., later exposure to that same virus). It could be the case that a small amount of mild stress during adolescence can prime systems to react more favorably to stressors in the future, rendering an adolescent resilient to the detrimental effects of stress. Studies are warranted that examine further whether stress during adolescence yields detrimental outcomes, or induces resilience.

1.3.1 Modeling adolescence in rodents

Animal models provide invaluable information regarding the human condition, both in the diseased and in the normally-functioning state. Investigators are granted precise experimental control in order to identify exact neurobiological mechanisms involved in a multitude of phenomena, including the unique period of adolescence. Rodent adolescence encompasses a much smaller timeframe compared to humans (days versus years), and thus serves as a useful model for understanding human adolescence, and how perturbations during this time impact functioning (Eiland & Romeo, 2013; Spear, 2000). In rodents, the increase in pubertal hormones resulting in preputial separation in males occurs at postnatal day (p) 42 ± 3 (Korenbrot et al., 1977; Vetter-O'Hagen & Spear, 2012). Spear's (2000) conservative definition of rodent adolescence from p28-42 is anchored around puberty and numerous behavioral changes. Others extend rodent adolescence to include periods just after weaning age, considered to be early adolescence/juvenility (p21-34), and periods post-puberty, considered to be late adolescence (p46-59) (Eiland & Romeo, 2013; McCormick & Mathews, 2007; McCormick et al., 2010; Tirelli et al., 2003).

1.3.2 Consequences of stress during adolescence

Much research has been concerned with understanding the effects of stress very early in life (either prenatally or within the first few weeks after birth), and the long-lasting consequences into adulthood [reviewed in: (Heim et al., 2004)]. Adolescence is now recognized as an important developmental time period; but most research on stress occurring at this time focuses on long-term consequences of stress, i.e., on the effects manifested in adulthood. Results of these

studies are variable, depending on stress protocol used and the sex of the animals tested, but tend to suggest impaired adult profiles as a result of adolescent stress exposure. For example, male rats exposed to severe sporadic stress during adolescence exhibited increased anxiety-related behaviors as adults (Pohl et al., 2007). Also, female, but not male, rats exposed to chronic mild stressors during adolescence exhibited anhedonia (decreased sucrose preference) and exaggerated corticosterone response to an acute stress as adults (Pohl et al., 2007). Finally, adolescent rats exposed to a mild repeated variable psychological/physical stress paradigm in adolescence had reduced exploration in an Open Field test later in young adulthood (Saul et al., 2012). Thus, it appears that stress during adolescence can lead to anxiety- and depressive-like phenotypes later in adulthood.

An important piece of information that warrants elucidation is the immediate effects of chronic stress during adolescence, i.e., the effects of stress during adolescence on *adolescents*. It could be that, although long-term consequences of adolescent stress exposure appear to be detrimental, those consequences develop as adolescence transitions into adulthood. If the systems impacted by stress have not yet been permanently altered, there could be an opportunity to provide protection from eventual detrimental effects of stress. Though sparse, there are some data on the immediate effects of chronic stress in adolescence. For example, pre-pubertal mice exposed to a series of variable stressors had decreased anxiety-like behavior and attenuated conditioned freezing behavior later in adolescence (Peleg-Raibstein & Feldon, 2011). Adolescent rats exposed to peri-pubertal psychogenic stress exhibited decreased anxiety-like behavior and increased risk-taking, with no change in depressive-like behavior or evoked corticosterone levels after an acute stress, when tested in late adolescence (Toledo-Rodriguez & Sandi, 2011). One of the only studies that utilized UCMS in adolescence found no difference in anxiety- or

depressive-like behaviors in adolescent rats, compared to Controls, and a separate group of animals exposed to the same procedures showed evidence for increased neurogenesis in the hippocampus (Toth et al., 2008). Together, this work suggests that adolescents may be resistant to depressive- and anxiety-like consequences of chronic stress in adolescence, but further studies are required that confirm this across behavior, neuroendocrine, and neural outcomes together in the same experiment. Considering the literature as a whole, critical information is still needed on the immediate effects of chronic stress exposure in adolescence.

1.4 UNPREDICTABLE CHRONIC MILD STRESS

Unpredictable chronic mild stress (UCMS) is the best validated paradigm for understanding the neurobiology of depression in adult rodents, at present (Konkle et al., 2003; Willner, 1997a, 2005). This paradigm was designed to mimic how humans often develop depression – adult rats or mice are exposed to a series of stressors that are mild in nature but that occur randomly over a period of 4-6 weeks. Adult rodents exposed to UCMS demonstrate behavioral symptoms of depression such as anhedonia (indicated by a decreased intake of sucrose solutions), and anxiety-like behaviors (evidenced in tests such as the Elevated Plus Maze and the Open Field test) (Lupien et al., 2009; Willner, 1997a, 2005). In addition to altering the behavioral phenotype, UCMS induces changes in functioning of the HPA axis and in the expression of certain proteins in the brain of adult rodents; these changes are similar to those seen in depressed humans (Adzic et al., 2009; Duman & Monteggia, 2006; Lupien et al., 2009; Toth et al., 2008). Specifically, UCMS in adult rats was found to decrease HPA axis negative feedback control, as seen in a dexamethasone suppression test, and to increase basal circulating levels of corticosterone

(Grippo et al., 2005a; Helmreich et al., 2008). UCMS and chronic variable stress paradigms in adult rodents were found to cause changes in brain systems that have also been demonstrated in depressed humans, including decreased phosphorylated CREB and phosphorylated ERK protein levels in the hippocampus, increased phosphorylated CREB in the nucleus accumbens, and down-regulation of the glucocorticoid receptor and regional atrophy in the hippocampus (Ferland et al., 2014; Grønli et al., 2006; Liu et al., 2014; Nestler et al., 2002) . Finally, consequences of UCMS exposure can be reversed with subsequent antidepressant treatment, indicating good predictive validity of this model (Willner, 1997a; Willner et al., 1994). Although data suggest that chronic stress in adolescence can induce depressive-like phenotypes later in adulthood (Avital et al., 2006; Avital & Richter-Levin, 2005; Lupien et al., 2009; McCormick et al., 2010; Toth et al., 2008), only a few studies have examined whether chronic stress *during adolescence* precipitates depressive-like behaviors *in adolescence* [reviewed in: (McCormick et al., 2010)].

1.5 INDIVIDUAL DIFFERENCES

Not all humans who are stressed go on to develop depression, anxiety, or addiction disorders. Differences in the way individuals respond to life experiences may determine whether or not they will develop neuropsychiatric disorders. Historically, variability in experimental data was treated as a nuisance, and often "aberrant" data were removed from analyses. Now, we see a shift in the field, with investigators acknowledging, and at times embracing, variability [e.g.: (Belin et al., 2011; Ersche et al., 2012; Flagel et al., 2010; Piazza et al., 1989; Stead et al., 2006; Tarter et al., 2012)]. Variations in response strategies, particularly with regard to response to stress, are being examined in the hopes of identifying those individuals most at risk to develop

neuropsychiatric disorders, including depression, anxiety, and drug addiction. Clinically, this translates to identifying particular personality traits that are associated with vulnerability to the aforementioned disorders (Ersche et al., 2012; Tarter et al., 2012). Once specific traits have been identified as putting individuals at heightened risk for a disorder (e.g., novelty-seeking may increase risk for addiction), the next critical step is to identify the underlying neurobiological mechanisms responsible for the vulnerability, and to establish causal links between the traits and disease risks. Researchers have begun to utilize animal models in order to understand the neurobiology of those traits associated with increased vulnerability to disease [e.g.: (Belin et al., 2011; Flagel et al., 2010; Piazza et al., 1989; Stead et al., 2006)].

Identifying factors that can predict future outcomes would be beneficial in distinguishing adolescent individuals most at risk – or those who may be resilient – to the effects of chronic stress exposure during adolescence. Although many investigators have confirmed the vulnerable state of brain systems during adolescence (McCormick & Mathews, 2007, 2010; McCormick et al., 2010; Spear, 2000), few studies have assessed whether individual differences in any of the domains discussed below translate to differing degrees of susceptibility to stress-induced general anxiety and depression at one end of the continuum, and risk-taking and addiction at the other, *in adolescents*. In the few studies in which locomotor response to novelty was examined in young rats, the investigators failed to determine whether initial locomotor rankings are stable across time [e.g.: (Clinton et al., 2008; Oztan et al., 2011b; 2011a)]. It is therefore critical to examine whether differential domains remain relatively constant across an animal's lifetime, to confirm establishment of a stable attribute, or "trait," that is suitable for use as an organizing variable.

1.5.1 Locomotor Response to Novelty

Novelty-seeking behavior can be defined as the increased exploration of novel situations or stimuli (Vidal-Infer et al., 2012; Zuckerman, 1979). Locomotor activity in a novel environment is considered to be an assay for novelty-seeking behavior in rodents [e.g.: (Calvo et al., 2011; Dellu et al., 1996; Jama et al., 2008; Kabbaj et al., 2000)]. Individual differences in locomotor responses to novelty have been found to correlate with differences in the tendency to develop stress-induced trait anxiety, and depressive-like, or addictive behaviors. Specifically, adult rodents that display very low levels of distance traveled in a novel environment over a 2hr Open Field test (Low Responders) have been shown to be prone to develop anxiety- and depressive-like behavior after stress, whereas those that exhibit very high levels of distance traveled (High Responders) have been shown to be prone to exhibit reduced inhibitory control, elevated risk-taking, and addictive-like behaviors (Belin et al., 2011; Calvo et al., 2011; Flagel et al., 2010; Stead et al., 2006; Stedenfeld et al., 2011). Notably, the increased locomotor activity observed in the High Responder animals is specific to novel environments, as there are no group differences observed when rats are tested in a familiar space (Dellu et al., 1996). Additionally, neuroendocrine stress reactivity differs between the High- and Low-Responder groups, in an interesting manner: High Responders actually exhibit increased and prolonged corticosterone release in a novel environment compared to Low Responders, but when exposed to an acute restraint stress, both groups exhibit similar responses (Dellu et al., 1996; Piazza et al., 1991).

This variation in behavior and neuroendocrine profile was first identified in outbred strains of rodents (Piazza et al., 1989), but now has been transformed into a genetic model as well. High- and Low-Responders have been selectively bred (within each response group) over many generations to yield a line of rats that exhibit the extreme ends of the population (Stead et

al., 2006). These specially bred rats have also been shown to differ along the anxiety/affective domains in the same way as their outbred counterparts: bred-Low Responders were more susceptible to UCMS, as indicated by the development of anhedonia (Stedenfeld et al., 2011); and bred-High Responders acquired cocaine self-administration more rapidly (Flagel et al., 2010). The use of genetic models of particular behavioral attributes provides the power to investigate gene x environment interactions. When outbred animals are screened for a behavior and then categorized based on distribution across the population, it may be more suitable to consider the construct being modeled as an "attribute." Alternatively, when utilizing a genetic model such as the bred High- and Low-responders, it may be appropriate to refer to the construct being modeled as a "trait."

1.5.2 Sign-and-Goal Tracking

An additional model of individual differences that has been linked to depressive- and addictive-like profiles is that of Sign-trackers versus Goal-trackers. In this model, the categorization stems from differential attribution of motivational properties, i.e., incentive salience, to external, versus reward, cues [reviewed in: (Meyer et al., 2012; Robinson et al., 2014)]. Briefly, rodents are trained in an appetitive Pavlovian approach task, where a neutral auditory stimulus (conditioned stimulus; CS) is paired with food delivery into a receptacle (unconditioned stimulus; US). Over subsequent pairings, rodents consistently learn to approach the receptacle upon presentation of the CS [e.g.: (Kirschmann et al., 2014)]. Rodents often emit head entries into the food receptacle upon hearing the CS; this is known as Goal-tracking. In some cases, a discrete stimulus, e.g., a lever insertion into the training chamber, is used instead of the auditory CS. Some rodents approach this cue, even when it is spatially separate from the location of the CS tracking; this is

known as Sign-tracking [reviewed in: (Flagel et al., 2009; Meyer et al., 2012; Robinson et al., 2014)]. Studies have implicated a risk for addiction in the Sign-tracker animals (Flagel et al., 2008; 2006; Uslaner et al., 2006). In addition to attributing motivational significance to an appetitive cue, Sign-trackers have also been shown to attribute motivational significance to *aversive* cues (Morrow et al., 2011). Thus, although models of individual differences in behavioral attributes do exist for identifying individuals most at risk for a disease, results may be less straight-forward than originally anticipated.

1.6 GOALS AND RELEVANCE OF THIS DISSERTATION

In summary, adolescence is a period of substantial change for many behaviors and brain circuitry, and perturbations in normal development at this time may be detrimental. Little is known about the immediate effect of UCMS or other chronic stressors on the physiological and neurobiological profiles of adolescent rats (McCormick & Mathews, 2010; McCormick et al., 2010; Toth et al., 2008). One of the goals of the present work therefore is to determine whether exposure to UCMS in adolescence leads to depressive- or anxiety-like behavioral, physiological, or neural profiles in adolescence (**Chapter 2**). Additionally, it is unclear whether differential environmental experiences and individual differences, such as those reflected in locomotor response to novelty, confer differential sensitivity to UCMS-induced effects in adolescence. Therefore, additional goals of the present work are to determine whether varied peri-weaning experiences (**Chapter 3**) or varied locomotor activity in a novel environment among adolescents (**Chapter 4**) can serve as predictors of sensitivity to UCMS exposure during adolescence.

2.0 UNPREDICTABLE CHRONIC MILD STRESS DURING ADOLESCENCE CAUSES HYPERACTIVITY, DECREASED ANXIETY, AND NEUROENDOCRINE CHANGES IN ADOLESCENT RATS

2.1 INTRODUCTION

Adolescence is broadly defined as "the gradual transition from childhood to adulthood" (Spear, 2000). Many social, emotional, and cognitive behaviors are developing during this time of increasing responsibility (Doremus-Fitzwater et al., 2010; Sisk & Foster, 2004; Sisk & Zehr, 2005; Spear, 2000). Adolescence does not have clear markers of its beginning and end because it is inherently transitional. It does include the distinct period of puberty (i.e., sexual maturation), but also extends beyond this timeframe (McCormick & Mathews, 2007, 2010; Sisk & Foster, 2004; Sisk & Zehr, 2005; Spear, 2000).

Characteristic behaviors of adolescence that are seen across species, including risk-taking and reward-seeking, may have adaptive values; these behaviors aid the organism in transitioning from a dependent child to an independent adult (Spear, 2000). The changes in behavior can often be attributed to maturational changes (e.g., myelination and synaptic pruning, increases or decreases of receptor levels, elaborations of projections, increases or decreases in neurons and glia) that occur in multiple brain areas including the prefrontal cortex, limbic circuitry, and the hypothalamic-pituitary-adrenal (HPA) axis (Cunningham et al., 2002; Doremus-Fitzwater et al.,

2010; Rubinow & Juraska, 2009; Sowell et al., 1999). When brain systems are rapidly changing, external stimuli such as stress could negatively interfere with neurodevelopment; thus adolescence is often thought of as a period of vulnerability [reviewed in: (Davey et al., 2008; Eiland & Romeo, 2013; Hollis et al., 2013; Spear, 2000)].

The goal of understanding how adolescents respond to stressors, and what the behavioral, neuroendocrine, and neural consequences of exposure to stress during adolescence are, is garnering more attention. Most studies to date aim at understanding the effects of adolescent stress manifested in adulthood, i.e., the long-term consequences of adolescent stress [reviewed in: (Hollis et al., 2013; McCormick & Green, 2013; McCormick et al., 2010)]. For example: brief or repeated exposure to a series of repeated mild, variable psychological or physical stressors during adolescence was found to induce anxiety-like behaviors, decrease exploratory behaviors, and reduce dopamine levels in the prefrontal cortex in adult rats (Avital & Richter-Levin, 2005; Baune et al., 2009b; Luo et al., 2014; Saul et al., 2012; Tsoory et al., 2007); exposure to chronic variable stress in mid- to late-adolescence was reported to cause hippocampal atrophy and reduced hippocampal glucocorticoid receptor gene expression in adult rats (Isgor et al., 2004); and exposure to a model of early life trauma (restraint, forced swim, ether inhalation) in early adolescence caused an increase in basal corticosterone levels and decrease in glucocorticoid receptor expression in the hippocampus in adult rats (Uys et al., 2006).

An important missing piece in our understanding of the consequences of adolescent stress is insight into how chronic stress in adolescence affects behavior, brain, and neuroendocrine function in adolescence. A few studies have addressed this issue; however, experimental protocols and results are varied. For example: brief intermittent peri-pubertal exposure to

psychogenic stress (predator odor, elevated platform exposure) was reported to decrease anxiety-like behavior and increase exploratory activity in male rats in late adolescence, with no apparent effects on depressive-like behavior or acutely-evoked corticosterone (Toledo-Rodriguez & Sandi, 2011). In contrast, Toth and colleagues (2008) found that exposure to chronic mild stress throughout adolescence had no effect on exploration, or anxiety- or depressive-like behaviors, but caused an increase in basal corticosterone levels in a separate group of animals; and chronic variable stress or chronic restraint stress during early-mid adolescence were reported to increase basal corticosterone levels in late adolescence (Lepsch et al., 2005).

Unpredictable chronic mild stress (UCMS) is the most validated paradigm for creating depressive- and anxiety-like phenotypes in adult rodents at present, in that it causes the key symptom of depression, anhedonia, and its effects can be reversed with chronic antidepressant treatment (Konkle et al., 2003; Willner, 1997a, 2005). The UCMS paradigm could be useful in examining the effects of chronic stress in adolescent rodents as well. Although numerous studies have examined how chronic stress in adolescent rats affects adult behavior, neuroendocrine, and neural function, only a few of them have employed UCMS paradigms (chronic mild stress, chronic unpredictable stress) (Pohl et al., 2007; Rincon et al., 2012; Toth et al., 2008), and even fewer have examined the immediate shifts in function, i.e., those that occur in adolescence during chronic stress (Toth et al., 2008).

UCMS exposure in adult rodents typically causes a decrease in weight gain, anhedonia as assessed by the sucrose preference test, an increase in anxiety-like behavior, and altered HPA axis function (both decreased HPA axis negative feedback control, as seen with the dexamethasone suppression test, and increased basal circulating levels of corticosterone) (Grippo et al., 2005a; Helmreich et al., 2008; Heydendael & Jacobson, 2010; Kunugi et al., 2010). Based

on the substantial literature on UCMS in adult rodents, and on the idea that adolescence is considered a period of heightened vulnerability to stress (Davey et al., 2008; Eiland & Romeo, 2013; Hollis et al., 2013; Spear, 2000), we reasoned that UCMS during adolescence would have particularly profound effects, resulting in depressive- and anxiety-like phenotypes accompanied by dysregulated HPA axis function, in adolescent rats. We exposed adolescent male rats to 5 weeks of UCMS starting in early adolescence, and evaluated behavioral, neuroendocrine, and neural consequences during the fifth week, i.e., in late adolescence.

2.2 MATERIALS AND METHODS

2.2.1 Animals

In this study, we defined rodent adolescence as a broad age range, starting one week post-weaning (postnatal day 28; p28), spanning through puberty, and ending around p60. This range included Spear's (2000) conservative definition anchored around puberty and behavioral changes (p28-42), as well as the period that is often considered to be late adolescence (Eiland & Romeo, 2013; McCormick & Mathews, 2007; Tirelli et al., 2003). Twenty adolescent male Sprague-Dawley rats (Charles River, Portage, MI) were used. Rats were weaned and shipped from the supplier on p21. Upon arrival, rats were housed in pairs in plastic cages (40 x 22 x 19cm). Standard rat chow (Purina) and water were available *ad libitum*, except when noted. Rooms were maintained on a standard 12hr light-dark cycle (lights on at 0700) except when noted, in a temperature and humidity controlled environment. Baseline sucrose preferences were determined on p30/31, and rats were divided into UCMS and Control groups (see descriptions below).

UCMS-exposed and Control groups were housed in separate rooms under similar conditions for the remainder of the study. All rats were weighed and observed daily. The same groups of rats were used for behavioral, physiological, and neurobiological assessments (Figure 2.1 and Table 2.1). The University of Pittsburgh Institutional Animal Care and Use Committee approved all procedures used.

2.2.2 Unpredictable Chronic Mild Stress (UCMS) Protocol

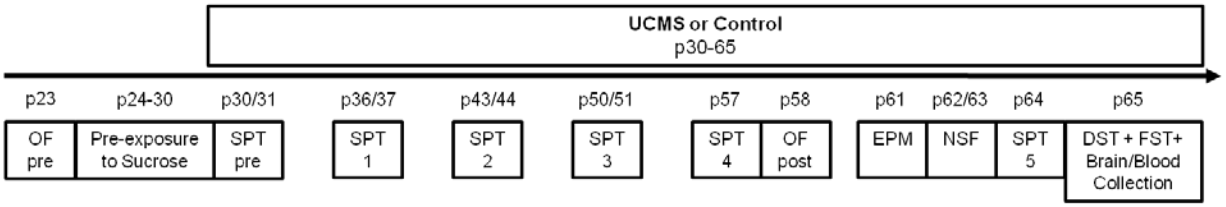
After an 8- to 9-day period of collecting baseline measurements (described below), adolescent rats (p30/31) were divided into UCMS (n = 10) and Control (Con; n = 10) groups. Briefly, UCMS rats were single-housed and exposed to a series of intermittent, mild stressors over the course of a week, for a total of five consecutive weeks (Figure 2.1A). This UCMS paradigm, modeled after Willner (1997a, 2005), was demonstrated to induce depressive-like behaviors in adult rats (Grippo et al., 2003; 2005a; 2005b; Grønli et al., 2004; Stedenfeld et al., 2011). Figure 2.1B shows an example of a typical week during UCMS. The following individual stressors were used in the UCMS protocol in varying order across different weeks: continuous overnight illumination; food restriction (2hr); overnight water deprivation (16hr) followed by 1hr of empty water bottle replacement; 45-degree cage tilt (6 or 18hr); stroboscopic lighting (6, 10, or 18hr; Eliminator Lighting mini-strobe E105; 8–10 flashes per second, 25W); overnight paired housing with another UCMS-exposed rat (18hr); damp/dirty bedding (300–500ml lukewarm water added to cage bedding; 18hr); white noise (3 or 5hr; radio static, 85dB); and predator odor exposure (10–60min exposure to 20µL undiluted 2,4,5-trimethylthiazoline (Contech Enterprises, Inc., Canada) placed onto a piece of gauze inside an open Eppendorf micro-centrifuge tube (Fisher Scientific, Pittsburgh, PA) and hung outside each cage). Control rats were pair-housed, and

underwent standard animal care throughout the course of the experiment (weekly cage changes and daily weighing). Controls were single-housed overnight once a week for the sucrose preference test (described below).

2.2.3 Behavioral Procedures

Baseline measures were taken prior to the start of UCMS. Assessments of weight occurred daily, assessments of anhedonia (sucrose preference test) occurred weekly, and behavioral tests of anxiety- and depressive-like behaviors were conducted during the last week of the five-week UCMS protocol. Rats underwent only one behavioral assessment per day (Figure 2.1A). Excluding the sucrose preference test (conducted in the housing rooms overnight), each behavioral test occurred during the light cycle in a single behavioral testing room. Rats were transported in their home cages and were allowed 5-10min to habituate to the room before the start of each test.

A



B

	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Sucrose Preference Test			5pm →	9am			
Food Deprivation		5-7pm		3-5pm			
Water Deprivation		5pm →	9am			6pm →	10am
Empty Water Bottle			9-10am				
Overnight Illumination	7am			7pm →	7am		7pm →
Cage Tilt	3:30pm →	9:30am			10am-4pm		
Paired Housing					4pm →	10am	
Damp Bedding					4pm →	10am	
Intermittent White Noise				4-9pm			12-3am
Stroboscopic Lighting		1-7am				8pm →	6am
Predator Odor	2:30-3pm						

Figure 2.1. Experimental details.

A) Experimental Timeline. Age in postnatal days (p) for each test is indicated. Adolescent rats arrived on p21 and baseline behavioral testing began two days later. UCMS: Unpredictable Chronic Mild Stress; OF: Open Field Test; SPT: Sucrose Preference Test; EPM: Elevated Plus Maze; NSF: Novelty-Suppressed Feeding Test; DST: Dexamethasone Suppression Test; FST: Forced Swim Test. B) Example of a typical week in the UCMS protocol. Rats were single-housed, and were exposed to 2-3 stressors each day.

Table 2.1. Experimental group sizes.

UCMS (n = 10) and Controls (n = 10) all were tested in a series of behavioral tests. On the final day of the experiment, rats were exposed to either BASELINE (n = 4 UCMS, 4 Con) or acute forced swim stress (FST; n = 6 UCMS, 6 Con) conditions. Finally, rats were either sacrificed by decapitation (n = 8 UCMS, 8 Con) for inclusion in Western blot analyses, or by perfusion (n = 2 UCMS, 2 Con) for future immunohistochemical analyses. FST: Forced Swim Test; DEX: Dexamethasone injection prior to FST; Saline: saline injection prior to FST.

Group (n)	**Behavior**	**End-Point**	**Brain Analyses**
Controls (10)	(10)	Baseline (4)	Western blot (4)
		Saline/FST (3)	Western blot (1) Immunohistochemistry (2)
		DEX/FST (3)	Western blot (3)
UCMS (10)	(10)	Baseline (4)	Western blot (4)
		Saline/FST (3)	Western blot (1) Immunohistochemistry (2)
		DEX/FST (3)	Western blot (3)

2.2.3.1 Open Field Test

The Open Field measures exploratory- and anxiety-like behavior in rodents (Brooks & Dunnett, 2009; Choleris et al., 2001; Coelho et al., 2014; Schmitt & Hiemke, 1998). It can also be used to specifically assess individual differences in stress response by measuring locomotor activity in response to a novel environment in a longer protocol (1-2hr exposure) (Calvo et al., 2011; Jama et al., 2008; Kabbaj et al., 2000; Stead et al., 2006; Stedenfeld et al., 2011). Rats were placed into an open chamber (43 x 43cm; MedAssociates, St. Albans, VT) under dim lighting (<10lux). Activity in the horizontal and vertical planes was monitored for two hours, via horizontal and vertical infared beam breaks across 16 evenly-spaced squares. Rats were tested on p23 (pre-stress), before assignment into UCMS or Con groups. Rats were tested again after 4 weeks of UCMS on p58 (post-stress). Because rats were tested multiple times in the Open Field, and novelty of the environment is important for the test, visual, tactile, and olfactory cues were altered between each Open Field exposure. The total distance traveled during the two hours provided an index of overall locomotor activity. Additional measures, such as time spent in the center, and amount of rearing were examined to provide an index of anxiety-like behavior (Schmidt & Duman, 2007; Schmitt & Hiemke, 1998).

2.2.3.2 Sucrose Preference Test

Rats were pre-exposed to having two drinking bottles in the home cage from p24-p29/30; one bottle had regular tap water and the other had sucrose solution (2% wt/vol; Fisher Scientific). On p29/30, baseline sucrose preference scores were taken: all rats were single-housed overnight with free access to two bottles placed in the center of the cage lid (one with regular tap water and one with 2% sucrose solution). Bottles were weighed pre- and post-test. Sucrose preference was quantified as the ratio of [total sucrose intake (g)] to [total fluid (sucrose + water) intake (g)] x

100. The morning after the baseline sucrose preference test (p30/31), rats were returned to paired housing. Rats then were randomly assigned to the Control and UCMS groups, with the restrictions that the average baseline sucrose preference and average locomotor activity scores during the pre-stress Open Field test were comparable between the two groups. UCMS rats then were single-housed in another room for the remainder of the study. Sucrose preference tests occurred once a week, with Control rats being shifted to single-housing one night per week during each sucrose preference test. Bottle position was counterbalanced across rats/weeks to avoid potential side biases.

2.2.3.3 Elevated Plus Maze

Rats were tested on the Elevated Plus Maze under dim lighting (open arms 5-10lux; closed arms <2lux) after 4 weeks of UCMS, on p61. Rats were placed, facing a closed arm, in the 10 x 10cm central area of a plus-shaped maze. Two of the fours arms (50 x 10cm) of the maze were open and two were closed. The maze was raised 83cm above the ground. The rats' exploratory activity was recorded for 5min and scored offline. Cumulative time and entries into open and closed arms were recorded, along with the latency to the first entry into an open arm (Walf & Frye, 2007).

2.2.3.4 Novelty-Suppressed Feeding Test

The Novelty-Suppressed Feeding test creates a conflict between the drive to eat and the fear of novel environments (Bessa, 2009; Bodnoff et al., 1989; Dulawa & Hen, 2005). Rats were tested on the Novelty-Suppressed Feeding test after 4 weeks of UCMS, on p62/63. Following ~24hr of food deprivation, rats were placed in one corner of a large, black, arena with slanted walls (outer diameter: 3 x 3ft; inner diameter: 50 x 50cm), under bright lighting (center ~800-900lux). The floor was covered in clean bedding, and a single food pellet was secured in the center of the

arena on a small, white, circular piece of Styrofoam. The latency to enter the center and latency to eat the food pellet were recorded and scored offline. The test ended after the rat took a bite of the secured food pellet or after 12min, whichever occurred first. Immediately following the Novelty-Suppressed Feeding test, food consumption in the home cage was recorded for 8min, to test whether motivation to eat was comparable between groups.

2.2.3.5 Object Investigation Test

A small subset of animals (n = 4 UCMS, 4 Con) was tested in an in-house object investigation task, to conduct a preliminary assessment for impulsive-like exploratory behaviors. One day after the Novelty-Suppressed Feeding test (p63), rats were individually placed into the familiar Novelty-Suppressed Feeding test arena, and were allowed to explore freely for 10min in dim light (corners and center ranged from ~45-60lux). Two objects were secured in diagonally-opposite areas of the arena (small jar with white lid, filled with white and colored beads; tall cylinder container filled with dark metal balls, with a thick black line around the center), and the floor was a smooth, clear, plastic insert. Behavior was videotaped and scored offline for latency to begin investigating the objects, and total number of contacts (sniffing/climbing on for ≥1s) with each object.

2.2.4 HPA Axis Analyses

2.2.4.1 Baseline Corticosterone

On the final day of the experiment (p65), some rats (n = 4 UCMS, 4 Con) received a single subcutaneous injection of 150mM NaCl (saline; 0.3ml) 6hr prior to sacrifice. Immediately before decapitation/perfusion, rats were transported in their home cage to the behavioral testing room,

and were anesthetized with an intraperitoneal injection of chloral hydrate (300mg/kg; Sigma-Aldrich, St. Louis, MO; dissolved in saline) or Fatal Plus (pentobarbital; 80mg/kg as 0.2ml/kg; Henry Schein Animal Health, Ohio). Brains and blood were then collected (described below).

2.2.4.2 Dexamethasone Suppression Test and Forced Swim Stress

The Dexamethasone Suppression Test examines for dysregulated HPA axis function: dexamethasone, a synthetic glucocorticoid agonist, is administered in order to suppress corticosterone release via negative feedback mechanisms (Helmreich et al., 2008). The forced swim test (FST) is a stress-based test that is sensitive to the behavioral effects of antidepressants (Castagné et al., 2011; Castagné et al., 2009; Porsolt et al., 1978; Schmidt & Duman, 2007); here we took advantage of this test as an acute stressor. On the final day of the experiment (p65), some rats received a single subcutaneous injection of either dexamethasone (DEX; 100µg/ml/1kg; University of Pittsburgh pharmacy; n = 3 UCMS, 3 Con) or vehicle (saline; 0.3ml; n = 3 UCMS, 3 Con), approximately 6hr prior to sacrifice. Immediately before decapitation/perfusion, rats were transported to the behavioral testing room in their home cage, and were given a 5min FST in a clear Plexiglas cylinder (~35cm high, 19in internal diameter; water ~20°C). Behavior was recorded and scored offline for latency to first immobile episode and total immobility time; immobility was defined as the absence of any movements except to keep nose above water for ≥1s. At the conclusion of the FST, rats were anesthetized, and brains and blood were collected (described below). The timing and dosage of DEX injection were based on published time-course and dose-response studies (Atkinson & Waddell, 1997; Helmreich et al., 2008; Lurie et al., 1989; Oxenkrug et al., 1984).

2.2.5 Tissue and Plasma Collection

On the final day of the experiment (p65), rats were anesthetized with an intraperitoneal injection of chloral hydrate between 4-6pm. Rats were decapitated, and brains were rapidly collected and flash frozen in isopentane (Acros Organics, Morris Plains, NJ); brains were stored at -80°C until later tissue analyses (see Western Blot Analyses, below). Trunk blood was also collected into ice-cold, heparin-coated tubes (BD Vacutainer 6ml tubes, sodium heparin 95USP units; Becton, Dickinson and Co., Franklin Lakes, NJ) at time of decapitation, and then centrifuged at 3,000rpm for 20min at 4°C; plasma was collected and stored at -80°C until later analyses (see ELISA Analyses, below). A small subset of rats (n = 2 UCMS, 2 Con) were transcardially perfused with saline followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.1M phosphate buffer, for future immunohistochemical analyses (see Table 2.1). These rats were anesthetized with an intraperitoneal injection of Fatal Plus (pentobarbital; 80mg/kg as 0.2ml/kg; Henry Schein Animal Health, Ohio) between 4-6pm. Blood was collected by aortic puncture immediately before perfusion and treated as described.

2.2.6 ELISA Analyses

As described above, plasma was collected and stored at -80°C until analysis of circulating corticosterone and interleukin-1β levels. Commercially-available ELISA kits were used, according to manufacturer's protocols, to assay for corticosterone (Immunodiagnostic Systems, Scottsdale, AZ) and interleukin-1β (R&D Systems, Minneapolis, MN). Coefficients of variation ranged from 0.01-0.20 (corticosterone) and 0.01-0.30 (interleukin-1β); average intra-assay

coefficient of variation was 0.12 (corticosterone) and 0.01 (interleukin-1β). Note that most interleukin-1β values fell below the lowest standard, 31.3pg/ml, and thus were undetectable.

2.2.7 Western blot Analyses

As described previously (Kirschmann et al., 2014; Remus & Thiels, 2013; Shiflett et al., 2008; 2009), tissue samples were excised from three consecutive 400µm-thick sections taken on a cryostat, either with a tissue punch (2mm in diameter; Fine Science Tools, Foster City, CA) or by hand-dissection. The following regions were collected: prefrontal cortex, basolateral nucleus of the amygdala, paraventricular nucleus of the hypothalamus, nucleus accumbens, dorsal striatum, dorsal hippocampus, and ventral hippocampus. Tissue samples were homogenized in a buffer containing 150mM NaCl, 1mM EDTA, 50mM Tris (pH 7.4), 0.05% SDS, 1% Triton-X-100, 1mM dithiothreitol, 2mM sodium fluoride, 1mM orthovanadate, 2mM sodium pyrophosphate, 100x protease inhibitor cocktail, and 1mg/ml pepstatin (Fisher Scientific; Sigma-Aldrich; BioRad, Hercules, CA; Millipore, Burlington, MA). Homogenate was centrifuged for 15min at 14,000rpm, the supernatant was collected, and protein concentration was determined in triplicates using a bicinchoninic acid assay (Pierce BCA Protein Assay, Fisher Scientific and 4% Copper(II) sulfate solution, Sigma-Aldrich). The samples were diluted to a uniform protein concentration with homogenization buffer and sample buffer containing 2.5M Tris (pH 6.8), 40% glycerol, 8% SDS, 0.05% bromophenol blue, and 30mg/ml dithiothreitol (BioRad, Fisher Scientific, Sigma-Aldrich), and then were heated to 37°C for 30min.

Forty-five µg of protein per sample were loaded for resolution by SDS-PAGE and subsequently electrophoretically transferred to Immobilon membranes (Fisher Scientific). The following proteins were assessed: phosphorylated cyclic adenosine monophosphate response

element-binding protein (pCREB), dopamine transporter (DAT), extracellular signal-regulated kinase1/2 (ERK), dual-phosphorylated, active ERK1/2 (pERK), glucocorticoid receptor (GR), phosphorylated glucocorticoid receptor (pGR), tyrosine hydroxylase (TH), and lactate dehydrogenase (LDH) or neuronal-specific nuclear protein (neuN) (protein loading controls). Membranes were probed for multiple proteins by cutting horizontally at various molecular weights. Membranes were blocked by incubating for 1hr at room temperature in a Tris-buffered saline (0.05M Tris [pH 7.9], 0.15M NaCl) plus 0.1% Tween 20 (Sigma-Aldrich) solution (TBST) containing 5% dried nonfat milk. Each membrane was incubated overnight with an antibody that specifically recognized the protein of interest: S-133 phosphorylated CREB (pCREB, 1:1000 dilution in 5% bovine serum albumin [BSA; dissolved in TBST]), DAT c-20 (1:1000 dilution in 2.5% milk), T-202/183- and Y-204/185-phosphorylated, i.e., active p44/42 MAPK (pERK, 1:2500 dilution in 5% BSA), GR p-20 (1:1000 dilution in 5% BSA), S-226-phosphorylated GR (pGRs226, 1:1000 dilution in 5% milk), TH (1:1000 dilution in 2.5% milk), or NeuN (1:5000 dilution in 5% BSA). Antigen binding was visualized with an HRP-linked secondary antibody (for pCREB, pERK, GR, pGRs226, TH: anti-rabbit, 1:5000 dilution in 5% milk; for NeuN: anti-mouse, 1:5000 dilution in 5% milk; for DAT: anti-goat, 1:10000 dilution in 5% milk) and an enhanced chemiluminescence reagent (Lumiglo; Cell Signaling, Beverly, MA). Blot images were captured with a CCD camera (Hamamatsu Photonics, Japan) and analyzed using densitometry software (UVP Labworks, Upland, CA). The pCREB and TH membranes then were stripped of antigen by incubation at 50°C for 45min in a solution containing 62.5mM Tris (pH 6.7), 2% SDS, and 0.62% β-mercaptoethanol, blocked in 5% milk, and incubated overnight with an antibody that specifically recognizes LDH (1:2000 dilution in 2% milk). Antigen binding (secondary antibody for LDH: anti-goat, 1:10000 dilution in 5% milk) was

visualized and blot images captured as described above. The pERK membranes were stripped of antigen as described, incubated overnight with an antibody that specifically recognizes phosphorylated and unphosphorylated, i.e., total p44/42 MAPK (tERK, 1:2500 dilution in 5% BSA), and antigen binding was visualized and blot images captured; membranes were stripped a second time and re-probed for LDH (see above). The pGRs226 membranes were stripped of antigen as described above, incubated overnight with antibody for S-211-phosphorylated GR (pGRs211, 1:1000 dilution in 5% milk), and antigen binding was visualized (secondary antibody for pGRs211: anti-rabbit, 1:5000 dilution in 5% milk), and blot images captured; membranes were stripped a second time and re-probed for total GR (see above). Antibodies were obtained from Abcam (pGRs211, pGRs226), Cell Signaling (pERK, tERK, anti-mouse, and anti-rabbit), Millipore (pCREB, NeuN, and TH), Rockland (anti-goat, LDH), and Santa Cruz (DAT and GR).

2.2.8 Statistical Analyses

For analyses of data collected throughout the UCMS exposure (weight, sucrose preference), scores were compared using repeated measures analyses of variance (ANOVAs) with group as the between-subjects factor and day as the within-subjects factor, followed by post-hoc pairwise comparisons using t-tests with Bonferroni's correction where necessary. For end-point behavior analyses, t-tests with group (UCMS, Con) as the between-subjects factor were conducted. Latency data were normally distributed, unless otherwise indicated; when necessary, data were transformed ($\log_{10}$) prior to statistical analyses. For Western blot analysis, ratios of GR to neuN immunoreactivity, pGRs211 or pGRs226 to GR immunoreactivity, pCREB to neuN immunoreactivity, pERK to tERK immunoreactivity, DAT to LDH immunoreactivity, or TH to LDH immunoreactivity were calculated for each sample in specific regions and then normalized

to Control values. Comparisons were made using t-tests for independent groups (UCMS, Con) for each protein of interest in each brain region. Where applicable, analyses were done separately for each end-point condition (BASELINE, FST). When group sizes fell below n = 5 (e.g., ELISA analyses, some Western blot analyses), nonparametric tests (Wilcoxon Rank-Sum) were conducted. Statistical analyses were performed using the SPSS software package version 20.0 (SPSS, Chicago, IL). Significance level was set to $\alpha \leq 0.05$.

2.3 RESULTS

2.3.1 UCMS during adolescence does not cause depressive-like behavior in adolescent rats

To determine the behavioral effects of UCMS during adolescence, we monitored weight and sucrose preference throughout the course of 5 weeks of UCMS, and we performed a series of behavioral assays during the fifth week of UCMS in late-adolescent rats. For reporting purposes, all behavioral results collected during the fifth week of UCMS will be referred to as "post-stress."

One hallmark feature of UCMS in adult male rodents is a substantial reduction in weight gain over time (Duncko et al., 2001; Guidotti et al., 2012; Konkle et al., 2003; Murison & Hansen, 2001; Willner et al., 1996). UCMS during adolescence also caused a significant blunting of weight gain. Figure 2.2A shows group average weights across the course of the experiment. Although rats from the two groups had similar weights pre-stress, adolescent rats in the UCMS group gained significantly less weight over time than did Control rats [main effect of group: $F(9,162) = 1598.58$, $p<0.001$]. UCMS rats weighed less than Controls by the third week of

UCMS, and this difference was maintained until the end of the study [post-hoc t-tests: all $p<0.05$]. Total weight gain (in grams) was significantly blunted in UCMS-exposed rats compared to Controls [$t(18) = 3.29$, $p=0.004$] (Figure 2.2B). When weight gain was expressed as percent change from baseline (calculated as [final weight – baseline weight] x 100), the groups remained statistically significantly different [$t(18) = 2.43$, $p=0.026$] (data not shown). These results indicate that the UCMS protocol used here during adolescence resulted in a persistent decrease in weight gain in adolescent male rats.

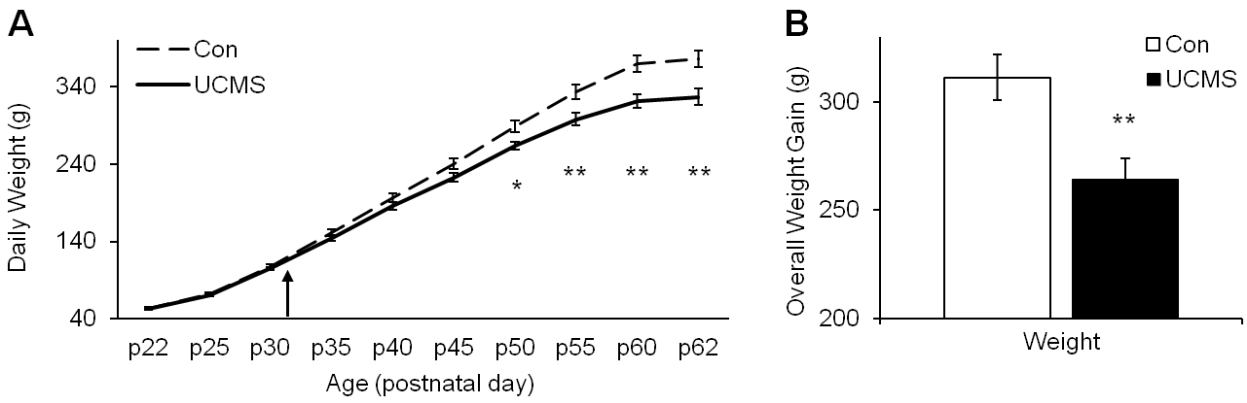


Figure 2.2. UCMS during adolescence blunts weight gain.

A) Daily weights in grams (g) throughout adolescence for rats in the UCMS group (n = 10) and the Control group (Con; n = 10). All rats gained weight during adolescence, but the weight gain was blunted in the UCMS group. Arrow indicates start of UCMS exposure. B) Total weight gained after 5 weeks of UCMS or Control conditions. Adolescent rats exposed to UCMS gained significantly less weight than did Controls. Overall weight gain (g) was calculated as [final weight – baseline weight]. Values show group means ± SEMs. **$p \leq 0.01$, *$p \leq 0.05$ for between-group comparisons.

We next examined whether there were depressive-like behavioral consequences in adolescent rats exposed to UCMS during adolescence. Adolescent controls preferred sucrose over water to a large degree (>75% preference) each week (Figure 2.3A). These data are consistent with what is typically reported in the literature (Grippo et al., 2003; 2005a; 2004; Stedenfeld et al., 2011). In contrast to the persistent stress effect on weight gain, UCMS during adolescence only transiently induced anhedonia, as measured by the sucrose preference test, in adolescent rats. Similar to controls, adolescent UCMS rats preferred sucrose over water to a large degree at baseline (Figure 2.3A). Although there was a significant main effect of group [$F(5,90) = 5.78$, $p<0.001$], there was also a significant group x sucrose preference test interaction [$F(5,90) = 2.97$, $p=0.016$]. UCMS sucrose preference scores were significantly reduced compared to controls only after one week of stress [post-hoc t-test: $p=0.003$]. This reduction in UCMS sucrose preference at week 1 was not only in comparison to control levels, but also in comparison to UCMS baseline [paired t-test: $t(9) = 2.28$, $p=0.049$] (Figure 2.3B). Reduced sucrose preference was not persistent: scores rebounded to control levels by week two of UCMS and were then maintained at those levels for the remainder of the UCMS [all $p>0.05$] (Figure 2.3A and 2.3B).

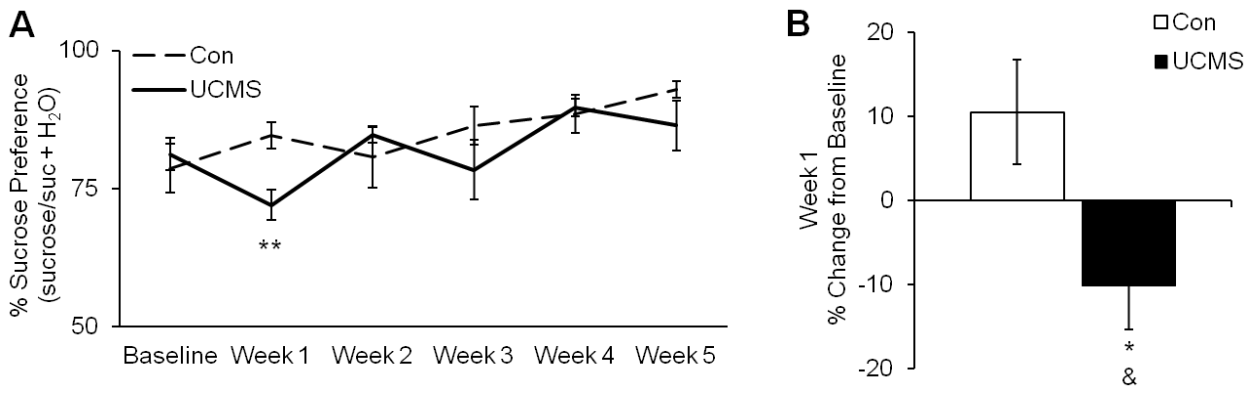


Figure 2.3. UCMS during adolescence does not cause long-lasting anhedonia.

A) Weekly sucrose preference scores throughout adolescence for rats in the UCMS group (n = 10) and the control group (Con; n = 10). Bottles of 2% sucrose and tap water were weighed pre- and post-test, and sucrose preference was quantified as [total sucrose intake (g) / total fluid intake (g)] x 100. Both groups preferred to drink sucrose. Despite a significant reduction in sucrose preference for the UCMS group after 1 week, preference levels equalized by week 2 and remained similar to controls for the rest of the study. B) Week 1 percent change in sucrose preference was calculated for each group as [(week 1 preference – baseline preference) / baseline preference] x 100. One week of UCMS did cause a significant reduction in sucrose preference, but the effect was not sustained. Values show group means ± SEMs. **$p \leq 0.01$, *$p \leq 0.05$ for between-group comparisons; & $p \leq 0.05$ for within-group comparisons to baseline.

Originally described by Porsolt (1978), the forced swim test (FST) examines for signs of behavioral despair in response to an acute stress by placing a rat into a container of water and then measuring the time it takes for the rat to "give up" and do nothing but float, as opposed to actively trying to escape (swimming/climbing behavior) [reviewed in: (Castagné et al., 2009; Cryan et al., 2005)]. We recorded the latency to begin floating and total immobility time in the acutely stressed UCMS and control rats. The FST yielded no signs of behavioral despair: UCMS-exposed adolescents did not differ from controls in latency to begin floating (Figure 2.4A) or total time spent immobile (Figure2.4B) [all $p>0.05$]. The UCMS group tended towards having slightly *longer* latencies to begin floating and *less* immobile time compared to controls, which suggests that they may exhibit more active coping skills than the controls, but neither measure reached statistical significance. Together, these results indicate that, although UCMS during adolescence induces a persistent reduction in weight gain, it does not induce depressive-like behavior as assessed by two standard tests – anhedonia in the sucrose preference test and behavioral despair in the FST – in adolescent rats.

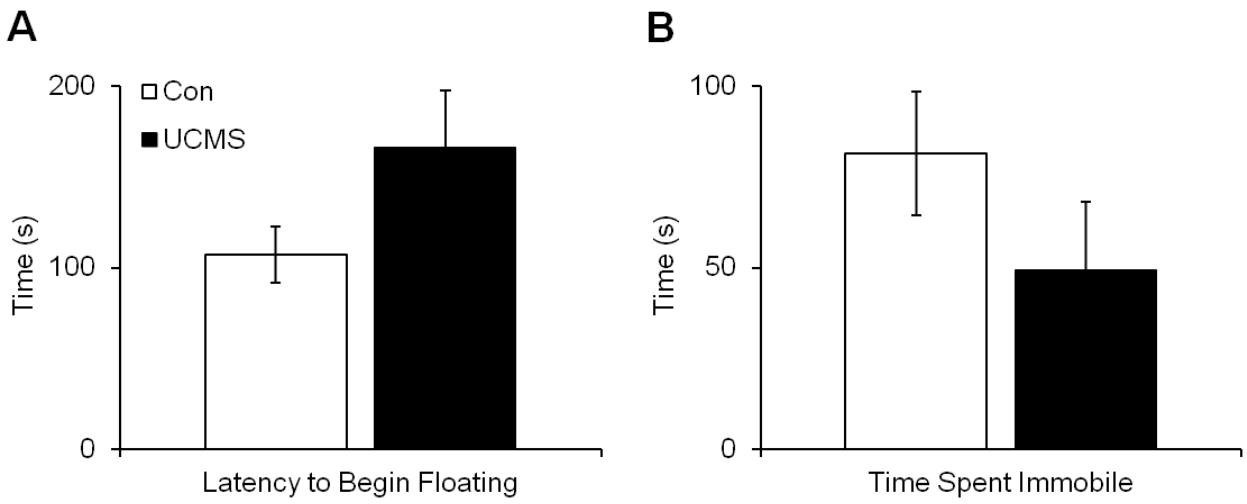


Figure 2.4. UCMS during adolescence does not induce behavioral despair.

Rats (n = 6 UCMS, 6 Con) were individually exposed to a 5min Forced Swim Test as an acute stressor prior to sacrifice. Behavioral despair was examined by recording A) latency to begin floating, and B) total time spent immobile. Though not statistically significant, adolescent rats exposed to UCMS tended to have a longer latency to begin floating and less time spent immobile compared to controls. Values show group means ± SEMs.

2.3.2 UCMS during adolescence causes hyperactivity, increased exploratory behavior, and decreased anxiety-like behavior in adolescent rats

Next, we assessed exploration and anxiety-like behaviors after UCMS during adolescence in the Open Field, Elevated Plus Maze, and Novelty-Suppressed Feeding tests. Regarding the Open Field test, a rat placed into a novel environment can be motivated by exploratory tendencies, emotional reactivity (e.g., anxiety), or both (Schmitt & Hiemke, 1998; Walsh & Cummins, 1976; Whimbey & Denenberg, 1967). Figure 2.5A shows locomotor activity, i.e., total distance traveled in the 2hr Open Field test before the start of UCMS (pre-stress) and during week 5 of UCMS (post-stress). There were main effects for test [$F(1,18) = 176.74$, $p<0.001$] and group [$F(1,18) = 28.34$, $p<0.001$]. Both groups increased total ambulatory distance in the Open Field post-stress, compared to the Open Field pre-stress. There was a significant group x test interaction [$F(1,18) = 31.98$, $p<0.001$]: although adolescent rats from the two groups displayed similar exploratory locomotor activity pre-stress, UCMS-exposed adolescents moved more over 2hr in the post-stress Open Field than did controls [post-hoc t-test: $p=0.004$]. Additionally, the percent increase in distance traveled from pre- to post-Open Field was significantly greater for UCMS rats compared to controls [$t(18) = 2.59$, $p=0.019$]. Figure 2.5B shows distance traveled in the Open Field post-stress after 15min. UCMS caused an increase in ambulatory distance that was evident within the first 15min [$t(18) = 3.35$, $p=0.004$]; the higher level of activity was then maintained throughout the 2hr test (as described above). Figure 2.5C shows vertical time (rearing) in the pre-stress and post-stress Open Field tests. Similar to the pattern observed with ambulatory distance, there were main effects for test [$F(1,18) = 55.00$, $p<0.001$] and group [$F(1,18) = 6.33$, $p=0.022$]. Both groups increased vertical time (rearing) in the Open Field post-stress, compared to the Open Field pre-stress. There was a significant group x test interaction

[F(1,18) = 5.73, p=0.028]: although adolescent rats from the two groups displayed similar time in the rearing position pre-stress, UCMS-exposed adolescents reared more over 2hr in the post-stress Open Field than did controls [post-hoc t-test: p=0.012]. In contrast to ambulatory activity, the UCMS-induced increase in vertical time was not evident at 15min [t(18) = 0.84, p>0.05] (Figure 2.5D) but instead emerged over the full 2hr test (as described above). When examining the total amount of time spent in the center of the Open Field arena, UCMS-exposed adolescent rats spent more time in the center than the controls during the full 2hr [t(18) = 3.31, p=0.004], a difference that developed over the course of the 2hr test, but was not evident during the first 15min [t(18) = 1.48, p>0.05] (Figure 2.5E). Examining the distribution of behaviors conducted in the center of the Open Field during the 2hr test (Figure 2.5F), we found that, despite spending more absolute time in the center compared to controls, the relative amount of time that UCMS-exposed adolescents allocated to various behaviors was strikingly similar to the relative distribution exhibited by the Con group. Both groups spent a majority of the time in the center resting (>40% for each), and divided the remaining time in the center somewhat evenly between fine motor (stereotypic) movements, rearing, and ambulating [repeated measures ANOVA: no main effect of group or group x behavior interaction, both p>0.05]. Together, performance in the Open Field suggests an upward shift in overall exploratory activity for UCMS-exposed adolescents, in addition to a downward shift in anxiety-like behavior.

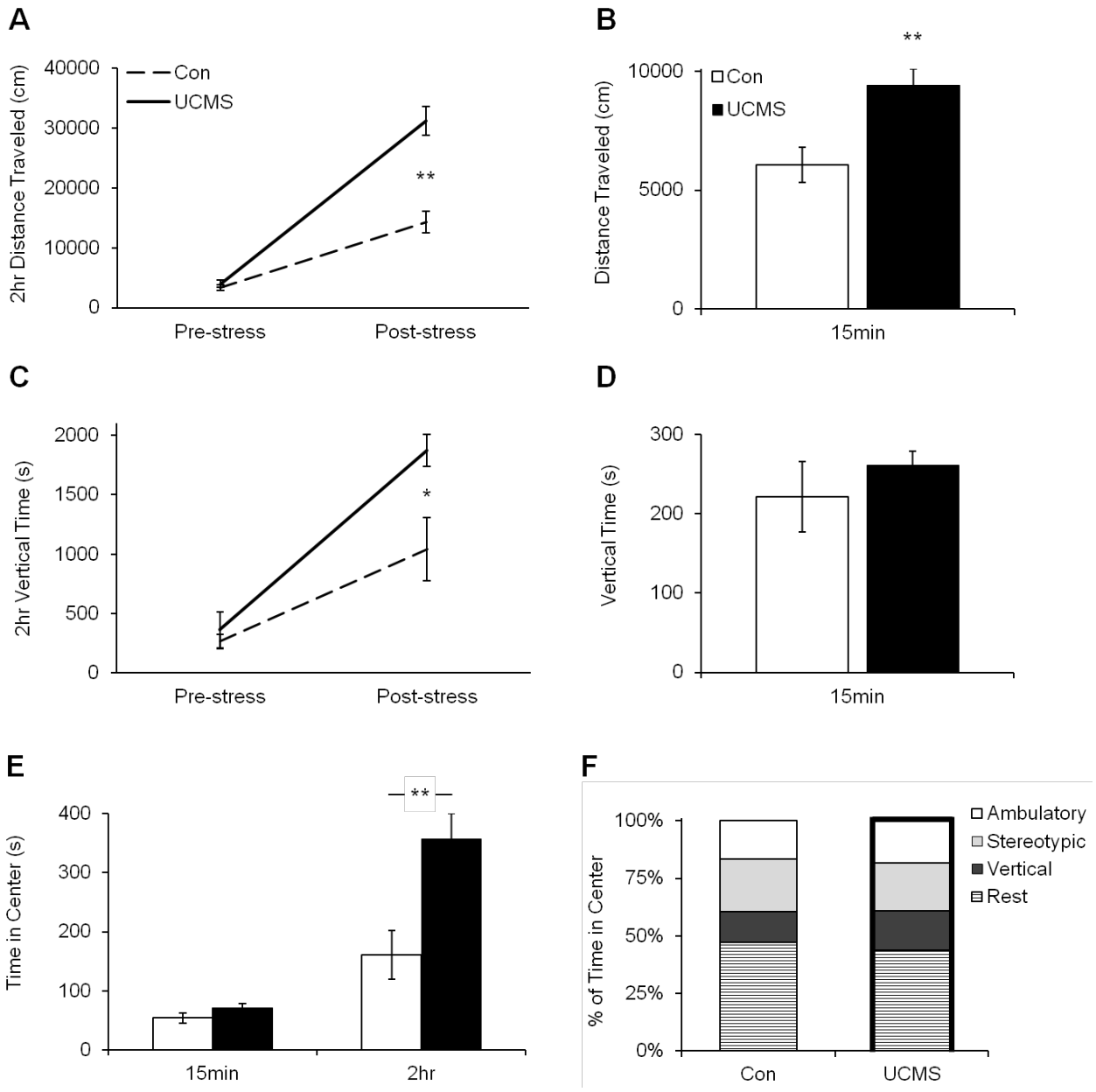


Figure 2.5. UCMS during adolescence causes hyperactivity and increased exploration in the Open Field Test.

A) Open Field Tests were given prior to the start of UCMS (Pre-stress) and during week 5 of UCMS during adolescence (Post-stress). Total distance traveled during the 2hr test did not differ between UCMS (n = 10) and controls (Con; n = 10) before the start of the stress exposure. Adolescent rats exposed to UCMS significantly increased ambulatory distance Post-stress. B) The increase in total distance traveled Post-stress in the UCMS group was present after 15min of the Open Field test. C) Vertical time (time spent rearing) in the Open Field test also was

similar between groups before stress, and significantly increased after UCMS exposure. D) The increase in vertical time Post-stress in the UCMS group was not evident at 15min. E) Adolescents exposed to UCMS spent more time in the center of the Open Field arena during the full 2hr test. F) Despite spending significantly more time in the center of the Open Field arena overall, UCMS and control rats had similar distributions of activities (percentage of time in center) including resting (rest), rearing (vertical), engaging in fine movements (stereotypic), and ambulating (ambulatory). Values show group means ± SEMs. **$p \leq 0.01$, *$p \leq 0.05$, for between-group comparisons.

We next examined anxiety-like behavior directly in the Elevated Plus Maze during week 5 of UCMS. Figure 2.6A shows the distribution of total, closed, and open arm entries; Figure 2.6B shows the percentage of open arm entries, relative to total number of entries; Figure 2.6C shows the amount of time spent in the open and closed arms; and Figure 2.6D shows the latencies to first enter open and closed arms after the start of the test. Consistent with the higher level of ambulatory activity noted in the Open Field, UCMS-exposed adolescents made more arm entries overall [$t(18) = 3.70$, $p=0.002$]. Consistent with lower levels of anxiety-like behavior noted in the Open Field, UCMS-exposed adolescents made significantly more open arm entries [$t(18) = 4.30$, $p<0.001$] than controls (Figure 2.6A). To correct for the difference in total arm entries between groups, we compared percentage of open arm entries (calculated as [number open arm entries/number of open + closed arm entries] x 100) (Figure 2.6B). Percentage of open arm entries was significantly higher in the UCMS group than the control group [$t(18) = 2.63$, $p=0.017$], indicating that the difference in absolute number of open arm entries between groups cannot be explained only in terms of hyperactivity by UCMS rats, but includes a downward shift in anxiety as well. Consistent with this interpretation, UCMS rats spent significantly more time in the open arms [$t(18) = 2.32$, $p=0.032$] compared to controls (Figure 2.6C). Latency to enter the open arms was not normally distributed and therefore we transformed the data ($\log_{10}$) prior to statistical analyses. The UCMS group first entered an open arm much more quickly than did the controls [$t(18) = 3.51$, $p=0.003$], which again suggests decreased anxiety-like behavior in this group (Figure 2.6D). Both groups made a similar number of closed arm entries [$t(18) = 1.28$, $p>0.05$] (Figure 2.6A) and entered the closed arm after a comparable amount of time [$t(18) = 1.25$, $p>0.05$] (Figure 2.6D). However, UCMS rats spent significantly less time in closed arms compared to controls [$t(18) = 2.09$, $p=0.051$] (Figure 2.6C). The Elevated Plus Maze data

indicate an increase in activity and a downward shift in anxiety in UCMS-exposed adolescent rats, consistent with the behavioral observations in the Open Field.

We also examined behavior in the Novelty-Suppressed Feeding test during week 5 of UCMS. The Novelty-Suppressed Feeding test creates a conflict between the drive to eat and the fear of novel environments, and has also been shown to be affected by antidepressant treatment, so it is often used to assess for anxiety- and depressive-like behaviors (Bessa, 2009; Bodnoff et al., 1989; Dulawa & Hen, 2005). There were no significant differences between groups for latency to enter the center of the arena (Figure 2.7A), time to eat the pellet (Figure 2.7B), or number of center entries/minute (Figure 2.7C) [all $p>0.05$]. There was a tendency for UCMS-exposed rats to begin eating the pellet sooner than controls, but this did not reach statistical significance. Motivation to eat was similar: home cage consumption of food, corrected for body weight, was comparable between groups [$t(18) = 0.69$, $p>0.05$] (Figure 2.7D). Thus, UCMS and Con groups exhibited similar behaviors in the Novelty-Suppressed Feeding test.

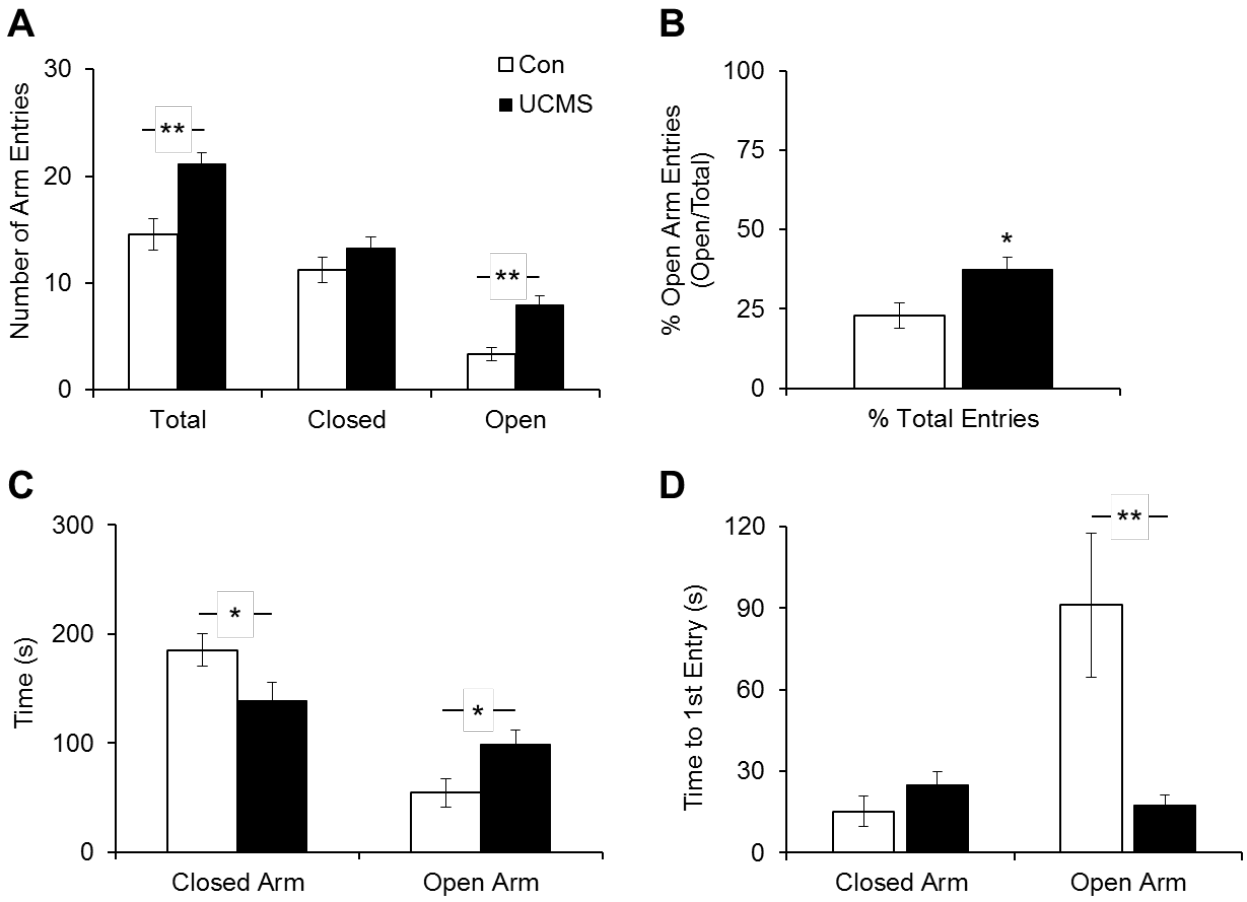


Figure 2.6. UCMS during adolescence causes hyperactivity and decreased anxiety in the Elevated Plus Maze.

A) Anxiety-like behavior was assessed in the Elevated Plus Maze during week 5 of UCMS. Adolescent rats exposed to UCMS (n = 10) developed hyperactivity, as evident by a significant increase in both open arm and total arm entries, compared to controls (Con; n = 10). B) To correct for the increase in total arm entries, the percentage of open arm entries was calculated as [open arm entries / total arm entries] x 100. The UCMS group displayed significantly more percent open arm entries than controls, which indicates a reduction in anxiety. C) Corresponding with number of arm entries, UCMS rats spent significantly more time in the open arms, and significantly less time in the closed arms, compared to controls. D) In addition to increased time on open arms, UCMS rats entered an open arm for the first time much faster than did controls. There were no differences in latency to first entry of a closed arm (after start of the test). Arm entries were scored when all 4 of the rat's paws had crossed over. Values show group means ± SEMs. **$p \leq 0.01$, *$p \leq 0.05$, for between-group comparisons.

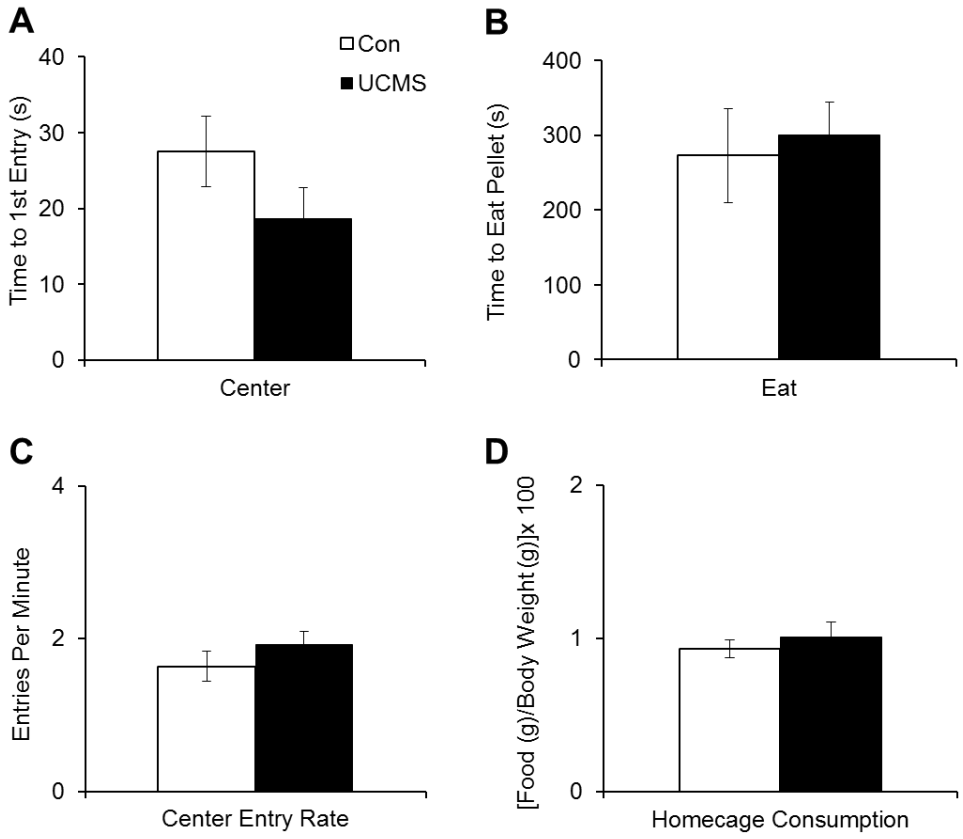


Figure 2.7. UCMS during adolescence does not alter behavior in the Novelty-Suppressed Feeding Test.

A) Behavior was also assessed in the Novelty-Suppressed Feeding test during week 5 of UCMS. Adolescent rats exposed to UCMS (n = 10) did not differ from controls (Con; n = 10) on any measures of this test, including latency to enter the center, or B) latency to eat the food pellet [both $p>0.05$]. C) Total number of entries into the center were recorded for the entire test. To correct for different lengths of test time for individual rats, the rate of center entries was calculated as [total number of entries into the center / total test time in seconds] x 60. There were no group differences in center entries per minute [$p>0.05$]. D) Home cage food consumption was corrected for body weight, and calculated as [food (g) / body weight (g)] x 100; home cage consumption was similar in both groups [$p>0.05$]. Values show group means ± SEMs.

Finally, to expand on our findings of hyperactivity and decreased anxiety in the Open Field and Elevated Plus Maze, we conducted a preliminary assessment for impulsive-like behaviors in a small subset of our experimental animals (n = 4 UCMS, 4 Con), We created an object investigation test, in which two objects were placed into a familiar open environment (Novelty-Suppressed Feeding test arena) and rats' investigation of the objects was monitored for 10min. Contacts with the objects lasting for ≥1s were recorded. Despite the small group size, there was a trend for the UCMS group to display more object contacts, compared to controls (Figure 2.8A). Acknowledging the small group sizes, we conducted non-parametric analyses on object contacts and confirmed this trend for increased object contacts by the UCMS group [Wilcoxon Rank-Sum (W) = 24, p=0.057]. Both UCMS and control groups investigated the first object within 3s of the start of the test (Figure 2.8B), but there was a trend for the controls to be slightly slower to examine the other object (W = 24, p=0.057). Although preliminary at best, these data of impulsive exploratory behaviors lend support to our hypothesis of increased exploratory behavior, in addition to a propensity to be hyperactive, in the UCMS group.

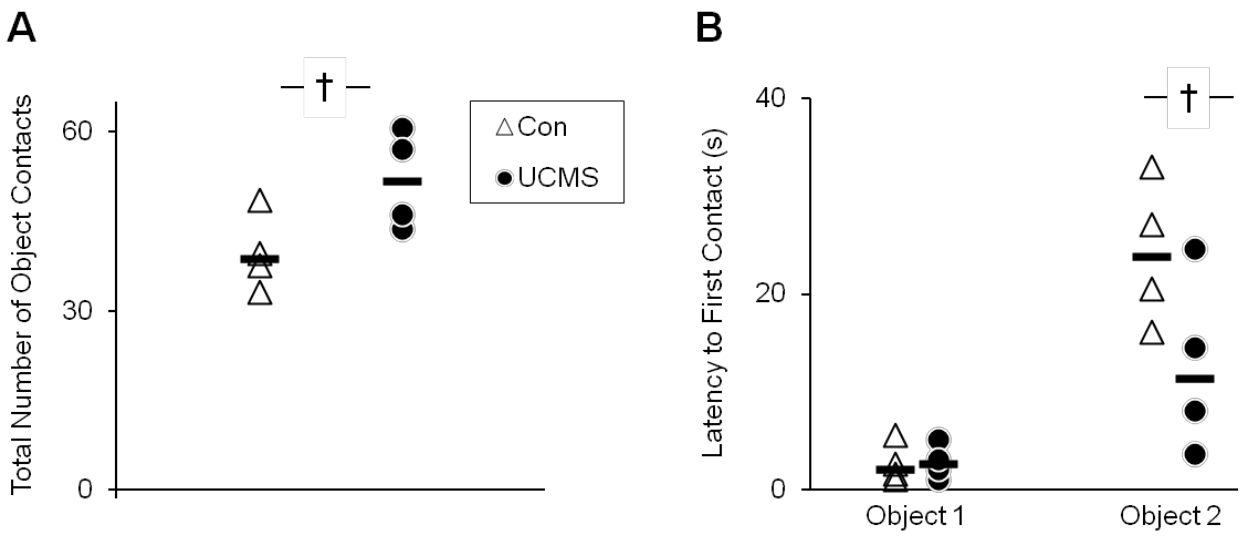


Figure 2.8. UCMS during adolescence increases object exploration in addition to increasing activity.

Impulsive exploratory-like behavior in a 10min object investigation test was examined more closely in a subset of UCMS (n = 4; circles) and Control (Con ; n = 4; triangles) rats during week 5 of UCMS. A) There was a trend for UCMS-exposed adolescent rats to make more investigatory contacts (sniffing/climbing on object for ≥1s) with two objects, compared to controls. B) This increased exploration was not solely due to increased locomotion of the UCMS group; both UCMS and control groups took similar amounts of time to being to investigate each object during the test. However, there was a trend for the UCMS group to explore the second object more quickly than controls. Graphs show individual data points, with group medians for each measure represented by horizontal bars. †$p \leq 0.06$ for between-group comparisons.

Together, our behavioral data indicate a unique profile in adolescent rats exposed to UCMS throughout the course of adolescence: there was a substantial reduction in weight gain, which is classically seen upon exposure to UCMS. In contrast to what has been reported in adults, UCMS during adolescence did not cause signs of anhedonia, behavioral despair, or an increase in anxiety. Instead, UCMS during adolescence caused a distinct hyperactive, exploratory, and low-anxiety behavioral profile in adolescent rats. We next aimed to link these behavioral changes with neuroendocrine changes.

2.3.3 UCMS during adolescence causes neuroendocrine changes but does not affect circulating cytokines in adolescent rats

To determine the effects of UCMS during adolescence on HPA axis function and circulating inflammatory cytokines, we collected plasma upon termination of the study after the fifth week of UCMS in the same late-adolescent rats that underwent all behavioral testing. Some rats (n = 4 UCMS, 4 Con) received a saline injection 6hr prior to sacrifice and served as the BASELINE group; some rats (n = 3 UCMS, 3 Con) received saline injections 6hr before undergoing an acute forced swim stress and served as the acute stress group (sal/FST); and some rats (n = 3 UCMS, 3 Con) received a dexamethasone injection 6hr prior to the acute forced swim stress and served as the negative feedback regulation group (DEX/FST). Acknowledging the small group sizes, we conducted non-parametric analyses. Figure 2.9 shows circulating corticosterone levels for rats in each treatment group. Adolescent rats exposed to UCMS had a different profile of corticosterone expression than controls, but showed no signs of impaired HPA axis function. Compared to the control group, UCMS-exposed adolescent rats had significantly lower levels of corticosterone at BASELINE [Wilcoxon Rank-Sum (W) = 25, p=0.029] (Figure 2.9A) and after an acute FST

stress [W = 15, p=0.05] (Figure 2.9B). UCMS had no effect on DEX-suppressed corticosterone levels, as indicated by the lack of a statistically significant difference between the groups [W = 11, p>0.05] (Figure 2.9C). Together, these results suggest that UCMS during adolescence shifts both basal and acutely-evoked corticosterone levels to lower levels than observed in controls, but it does not impair HPA axis function. Additionally, the negative feedback mechanisms of the HPA axis do not appear to be impaired. However, because of the overall lower levels of corticosterone in UCMS-exposed adolescents and the small group sizes, we cannot rule out that a floor effect prevented us from detecting a possible over-activation of the negative feedback system.

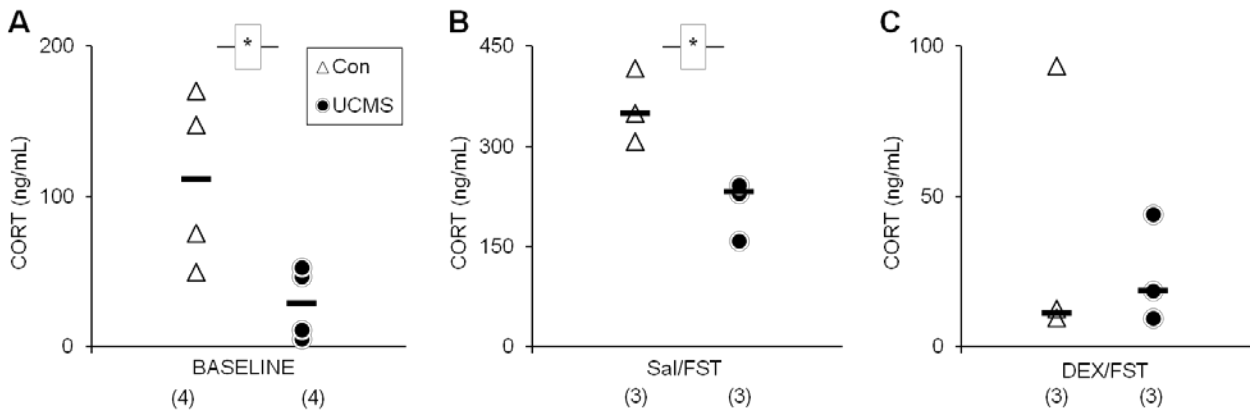


Figure 2.9. UCMS during adolescence lowers basal and evoked corticosterone, but does not impair overall HPA axis function or negative feedback.

HPA axis function was assessed after 5 weeks of UCMS (n = 10; circles) or control conditions (n = 10; triangles) in the same rats from the behavioral experiments. Despite a shift in neuroendocrine levels, there was no evidence of abnormal HPA axis function. Graphs show individual corticosterone levels (ng/ml) for each rat in its appropriate condition, with group medians for each treatment represented by horizontal bars. Please note the different scales in each panel. A) BASELINE rats (n = 4 UCMS, 4 Con) received saline injections 6hr before being sacrificed, to assess basal circulating corticosterone levels. Adolescent rats exposed to UCMS had significantly lower basal corticosterone compared to controls. B) Some rats (n = 3 UCMS, 3 Con) received saline injections 6hr before undergoing an acute forced swim test (sal/FST) immediately before sacrifice, to assess acute HPA axis response. Adolescent rats exposed to UCMS had significantly lower evoked corticosterone levels compared to controls. C) Finally, some rats (n = 3 UCMS, 3 Con) received dexamethasone injections 6hr before undergoing an acute forced swim test (DEX/FST) immediately before sacrifice, to assess negative feedback regulation of the HPA axis. Negative feedback regulation did not appear to be blunted in UCMS-exposed rats. *$p \leq 0.05$, for between-group comparisons.

Chronic stress has also been shown to alter levels of circulating inflammatory cytokines [(Grippo et al., 2005a); reviewed in: (Goshen & Yirmiya, 2009)]. Some work using specially-bred rats with increased vulnerability to UCMS demonstrated a four-fold increase in plasma interleukin-1β in adult rats after UCMS, that was attenuated by antidepressant treatment [(Stedenfeld, Sved & Saavdra, unpublished observations), (Stedenfeld, 2011)]. We examined plasma interleukin-1β in our adolescent Con and UCMS groups, and did not detect an increase in cytokine levels after UCMS (data not shown).

2.3.4 UCMS during adolescence does not alter stress- or emotion-related proteins in limbic circuitry, but does alter stress-related proteins in the paraventricular nucleus in adolescent rats

We performed region-specific Western blot analyses on brains collected from UCMS (n = 8) and control rats (n = 8) after the final behavioral procedure (BASELINE or acute stress) was completed. We examined changes in proteins related to stress, emotion/anhedonia, and activity, as discussed below. When examining phosphorylated proteins, it was necessary to separate the UCMS and control groups into BASELINE and acutely-stressed (FST) subgroups, because the phosphorylation state of these proteins can be influenced by acute stress, such as caused by the FST (Yang et al., 2008; 2004; Zheng et al., 2008). Thus, group sizes often were reduced to 4-6 rats per group, and nonparametric analyses were conducted accordingly.

Chronic stress and early life trauma have both been shown to cause reductions in hippocampal glucocorticoid receptor levels and hippocampal atrophy in adults (Herman et al., 1995; Isgor et al., 2004; Ladd et al., 2004; Magarinos & McEwen, 1995; Uys et al., 2006). Early life environmental disturbances have been shown to cause a reduction in glucocorticoid receptor

levels in the paraventricular nucleus as well (Avishai-Eliner et al., 2001; Gilles et al., 1996). Therefore, we predicted that UCMS exposure would cause a decrease in hippocampal and paraventricular nucleus glucocorticoid receptor levels in UCMS-exposed adolescent rats. Table 2.2 summarizes our glucocorticoid receptor findings. Different from our predictions, we saw no change in total glucocorticoid receptor levels (glucocorticoid receptor immunoreactivity relative to neuN immunoreactivity) in the dorsal hippocampus, ventral hippocampus, or paraventricular nucleus [all $p>0.05$].

Table 2.2. UCMS during adolescence does not alter glucocorticoid receptor levels in the paraventricular nucleus or hippocampus.

UCMS (n = 8) and Con (n = 8) brains were analyzed via Western blot for levels of glucocorticoid receptor in the paraventricular nucleus of the hypothalamus (PVN), dorsal hippocampus (DHipp), and ventral hippocampus (VHipp). Glucocorticoid immunoreactivity was corrected for total protein loading (neuN) immunoreactivity. Values expressed are group means ±SEMs of the ratios of [GR/neuN] normalized to control values for each region.

Protein	Brain Region	Con	UCMS
GR/neuN	**PVN**	1.0 (0.10)	0.8 (0.06)
	DHipp	1.0 (0.09)	0.9 (0.08)
	VHipp	1.0 (0.03)	1.1 (0.06)

Glucocorticoid receptor function can be regulated by phosphorylation on numerous serine residues in the N-terminal domain (Chen et al., 2008; Krstic et al., 1997). Two sites of particular interest are serine-211 (GRs211) and serine-226 (GRs226): the relative level of phosphorylation at s211 versus phosphorylation at s226 has been shown to be a determinant of glucocorticoid receptor transcriptional activation, such that when relative levels of s211 phosphorylation exceed relative levels of s226 phosphorylation, there is increased glucocorticoid receptor transcriptional activation and increased expression of glucocorticoid receptor-responsive genes, and vice versa (Blind & Garabedian, 2008; Chen et al., 2008). Chronic mild stress in adult rats caused a significant decrease in glucocorticoid receptor s211-phosphorylation in the prefrontal cortex (Guidotti et al., 2012). Chronic isolation stress caused increased glucocorticoid receptor s211-phosphorylation and decreased glucocorticoid receptor s226-phosphorylation in the hippocampus, in conjunction with decreased circulating corticosterone [(Adzic et al., 2009); reviewed in: (Galliher-Beckley & Cidlowski, 2009)]. In a subset of depressed patients that showed elevated levels of cortisol, Simic and colleagues found *increased* levels of glucocorticoid receptor s226-phosphorylation, and a decreased ratio of glucocorticoid receptor s211/s226 phosphorylation (as assessed by peripheral blood mononuclear cells) (2013). We examined the effect of UCMS on s211- and s226-phosphorylated glucocorticoid receptor levels in the hippocampus and in the prefrontal cortex. Table 2.3 details the results from analyses of glucocorticoid receptor phosphorylation state. Under BASELINE conditions, there was a trend for phosphorylation of glucocorticoid receptor on serine-211 (s211-phosphorylated glucocorticoid receptor immunoreactivity relative to total glucocorticoid receptor immunoreactivity) to be lower in the dorsal hippocampus of UCMS rats compared to control rats [$W = 24$, $p=0.057$]. However, neither under BASELINE conditions nor after the acute stress

induced by FST did the phosphorylation state at the respective residues differ between UCMS or Con in either the prefrontal cortex or the ventral hippocampus, and the trend of reduced glucocorticoid receptor s211-phosphorylation observed in the dorsal hippocampus of UCMS rats under baseline conditions was absent after FST. There also were no differences in the ratio of glucocorticoid receptor s211-phosphorylation relative to s226-phosphorylation in any of the regions analyzed [all $p>0.05$]. Thus, together with the total glucocorticoid receptor data, our findings suggest that UCMS during adolescence does not substantially alter total glucocorticoid receptor levels or function in key areas involved in regulation of the stress response in adolescent rats.

Table 2.3. UCMS during adolescence does not alter phosphorylation state of glucocorticoid receptor.

UCMS (n = 8) and controls (n = 8) were separated by end-point condition: BASELINE (n = 4 UCMS, 4 Con) and FST (n = 4 UCMS, 4 Con). Brains were analyzed via Western blot for levels of glucocorticoid receptor phosphorylation at serine-211 (pGRs211) and serine-226 (pGRs226) in the prefrontal cortex (PFC), dorsal hippocampus (DHipp), and ventral hippocampus (VHipp). There was a trend at BASELINE for UCMS rats to have lower glucocorticoid receptor s211-phosphorylation in the dorsal hippocampus (values shown in bold). Phosphorylated glucocorticoid receptor immunoreactivity was corrected for total protein (glucocorticoid receptor) immunoreactivity. Values expressed are median (range) ratios of [pGR/GR] normalized to BASELINE control values for each region.†$p \leq 0.06$ for between-groups comparisons.

Condition	Brain Region	pGRs211/GR		pGRs226/GR	
		Con	UCMS	Con	UCMS
BASELINE	**PFC**	1.0 (0.3-1.4)	1.0 (0.5-1.6)	1.0 (0.8-1.1)	1.0 (0.8-1.1)
	DHipp	**1.0 (0.9-1.1)**	**0.8 † (0.6-1.0)**	1.0 (0.9-1.2)	0.9 (0.8-1.2)
	VHipp	1.0 (0.9-1.2)	0.9 (0.4-1.1)	1.0 (1.0-1.1)	1.0 (0.9-1.2)
FST	**PFC**	0.8 (0.3-1.8)	1.3 (0.9-1.6)	1.0 (0.7-1.5)	1.1 (0.9-1.5)
	DHipp	1.0 (0.9-1.2)	1.0 (0.8-1.1)	1.1 (0.8-1.3)	1.0 (0.8-1.3)
	VHipp	1.3 (0.7-1.7)	0.8 (0.5-1.1)	1.2 (1.0-1.3)	1.1 (0.7-1.2)

Stress has also been shown to alter the extracellular signal-regulated kinase 1/2 (ERK) pathway, although results are varied. For example, ERK2 phosphorylation was reduced in the hippocampus after unpredictable restraint/shock or chronic forced swim stress (Jeon et al., 2012; Qi et al., 2008; 2006). Hippocampal ERK1/2 phosphorylation was also reduced after direct exposure to corticosterone for 2 weeks (Gourley et al., 2008b). On the other hand, others have reported acute stress exposure to *increase* ERK1/2 phosphorylation in the hippocampus and the basolateral nucleus of the amygdala (Yang et al., 2008; 2004; Zheng et al., 2008). Additionally, systemic inhibition of ERK 1/2 activation using an inhibitor of the ERK kinase mitogen-activated protein kinase/ERK kinase (MEK) has been linked to hyperactivity in the Open Field test and decreased anxiety in the Elevated Plus Maze (Einat et al., 2003). Together, these data warranted an examination of whether there are changes in ERK1/2 activation in the basolateral nucleus of the amygdala and hippocampus in our UCMS-exposed adolescents. However we found no significant group differences in ERK1/2 activation (phosphorylated ERK1/2 immunoreactivity relative to total ERK1/2 immunoreactivity) in the hippocampus or the basolateral nucleus of the amygdala (Table 2.4). Overall, there were no substantial shifts in ERK1/2 activation in limbic circuitry after UCMS during adolescence [all $p>0.05$].

Table 2.4. UCMS during adolescence does not alter ERK activation in limbic circuitry.

UCMS (n = 8) and controls (Con; n = 8) were separated by end-point condition: BASELINE (n = 4 UCMS, 4 Con) and FST (n = 4 UCMS, 4 Con). Brains were analyzed via Western blot for levels of dual-phosphorylated p44/p42 ERK (pERK1/2) in the basolateral nucleus of the amygdala (BLA), dorsal hippocampus (DHipp), and ventral hippocampus (VHipp). Phosphorylated ERK1/2 immunoreactivity was corrected for total protein (ERK1/2) immunoreactivity. Values expressed are median (range) ratios of [pERK1/2 / ERK1/2] normalized to BASELINE control values for each region.

Condition	Brain Region	pERK1/2 / tERK1/2	
		Con	UCMS
BASELINE	**BLA**	1.0 (0.8-1.3)	1.0 (0.6-1.3)
	DHipp	1.0 (0.9-1.2)	1.1 (0.4-1.4)
	VHipp	1.0 (1.0-1.1)	0.9 (0.7-1.1)
FST	**BLA**	1.0 (0.6-1.4)	1.4 (0.7-1.6)
	DHipp	1.0 (0.7-2.1)	1.4 (1.1-1.5)
	VHipp	1.0 (0.9-1.1)	1.1 (1.0-1.6)

Interestingly, cyclic-AMP response element-binding protein (CREB), in conjunction with co-activator proteins, has been implicated as necessary for the transcription of corticotrophin-releasing hormone in the paraventricular nucleus after stress (Liu et al., 2011; Seasholtz et al., 1988). We therefore examined levels of CREB phosphorylated at the transcriptionally-relevant serine-133 site in the paraventricular nucleus and found significant group differences that depended on end-point condition. Phosphorylated CREB levels in the paraventricular nucleus did not differ between UCMS and controls at BASELINE, but were significantly lower in the paraventricular nucleus of UCMS- compared to controls after an acute stress (FST) [$W = 25$, $p=0.029$] (Figure 2.10). This blunting of phosphorylated CREB in the paraventricular nucleus after an acute stress, in combination with the lowered levels of corticosterone we reported earlier, suggests that UCMS during adolescence may be protective towards additional stress exposure.

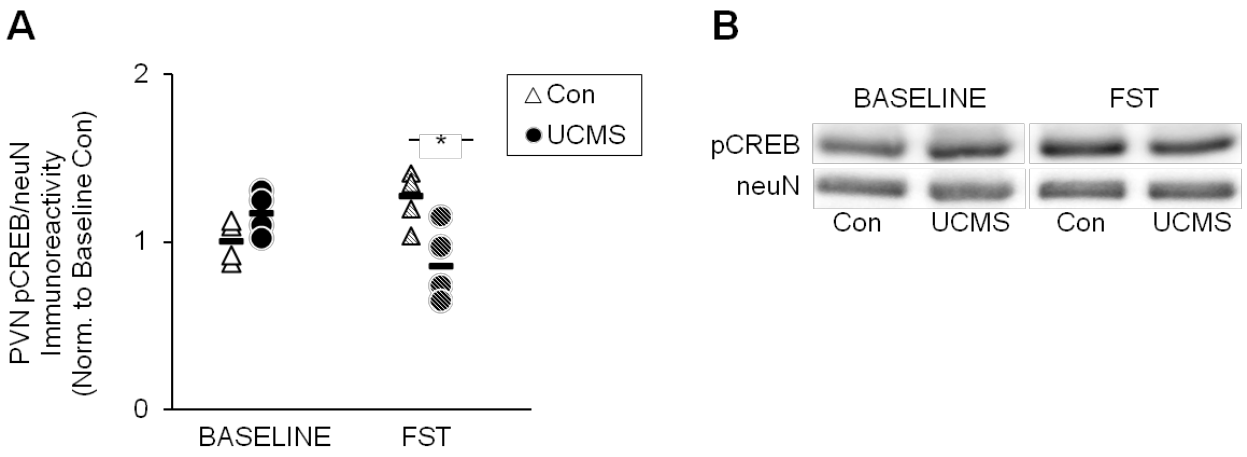


Figure 2.10. UCMS during adolescence differentially alters pCREB levels in the paraventricular nucleus.

Paraventricular nucleus phosphorylated CREB [pCREB immunoreactivity / neuN immunoreactivity] levels were examined via Western blot of tissue collected from UCMS (n = 8; circles) and control (Con; n = 8, triangles) groups after the last behavioral procedure. A) At BASELINE, UCMS (n = 4) and control (n = 4) adolescents had similar levels of phosphorylated CREB (pCREB) in the paraventricular nucleus. After an acute FST stress, UCMS adolescents (n = 4) had significantly lower levels of pCREB in the paraventricular nucleus compared to controls (n = 4). Individual rats' data are plotted as symbols for BASELINE (solid symbols) and FST (hashed symbols) groups and are normalized to BASELINE control values; horizontal bars indicate group median values. *$p \leq 0.05$ for between-group comparisons. B) Example blots of pCREB and neuN from the paraventricular nucleus of control and UCMS rats in the BASELINE or FST conditions.

Depression has been associated with changes in levels of CREB phosphorylation in limbic/reward circuitry: both *post mortem* tissue from depressed humans and tissue collected from rodents that exhibited symptoms of depression (including anhedonia) have shown increases of CREB phosphorylation in the nucleus accumbens, coupled with decreases of CREB phosphorylation in the hippocampus [(Muschamp et al., 2011); reviewed in: (Nestler et al., 2002; Russo & Nestler, 2013)]. Phosphorylation of CREB in the nucleus accumbens was also shown to be increased in response to emotionally-salient stimuli in general (Barrot et al., 2002; Muschamp et al., 2011; Tropea et al., 2008). Although we did not see much evidence for depressive-like behaviors after UCMS exposure during adolescence, we wanted to determine whether the brains of UCMS-exposed adolescents lacked depressive-like changes as well. Complementing our data of no behavioral signs of depression, we found no changes in phosphorylated CREB (pCREB immunoreactivity relative to neuN immunoreactivity) in the nucleus accumbens or either hippocampal region at BASELINE [all $p>0.05$] (Table 2.5). After an acute FST, pCREB levels in the dorsal hippocampus were significantly lower in UCMS rats than control rats [$W = 25$, $p=0.029$]. These findings again suggest that UCMS-exposed adolescents may be protected from the detrimental effects of additional stressors.

Table 2.5. UCMS during adolescence does not alter basal levels of pCREB in limbic circuitry.

UCMS (n = 8) and Con (n = 8) were separated by end-point condition: BASELINE (n = 4 UCMS, 4 Con) and FST (n = 4 UCMS, 4 Con). Brains were analyzed via Western blot for levels of s133-phosphorylated CREB (pCREB) in the nucleus accumbens (NAc), dorsal hippocampus (DHipp), and ventral hippocampus (VHipp). Phosphorylated CREB immunoreactivity was corrected for total protein loading (neuN) immunoreactivity. Values expressed are median (range) ratios of [pCREB/neuN] normalized to BASELINE control values for each region. *p<0.05 for between-group comparisons.

Condition	Brain Region	pCREB/neuN	
		Con	UCMS
BASELINE	**NAc**	1.0 (0.7-1.8)	0.8 (0.4-1.0)
	DHipp	1.0 (0.8-1.2)	1.1 (0.9-1.4)
	VHipp	1.0 (0.9-1.0)	1.1 (0.9-1.3)
FST	**NAc**	0.9 (0.4-1.4)	0.9 (0.8-1.6)
	DHipp	**1.1 (0.9-1.5)**	**0.8 * (0.7-1.0)**
	VHipp	0.9 (0.9-1.1)	1.0 (0.9-1.1)

2.3.5 Hyperactivity induced by UCMS exposure during adolescence is accompanied by changes in ERK activation, but not changes in the dopaminergic system, in the striatum

Adolescent rats have been shown to have lower expression of tyrosine hydroxylase in the dorsal striatum, compared to adults (Matthews et al., 2013). In that study, the lower levels of dorsal striatal tyrosine hydroxylase also correlated with lower levels of amphetamine-induced activity in an open field for the adolescents, as compared to adults (Matthews et al., 2013). Thus, there was a correlation with locomotor activity and dorsal striatal tyrosine hydroxylase levels. The dopamine transporter in the nucleus accumbens has also been shown to correlate with locomotor activity: drugs that interfere with dopamine uptake (e.g., psychostimulants) increase locomotor behavior, and dopamine transporter knockout mice exhibit hyperactivity in an Open Field (Beaulieu et al., 2006; Gainetdinov, 1999; Giros et al., 1996). Our behavioral results demonstrated an increase in activity after UCMS exposure in adolescence. Given that the dopaminergic systems are rapidly developing during the period of adolescence, and relationships between activity and both tyrosine hydroxylase and the dopamine transporter have been demonstrated, we measured tyrosine hydroxylase and dopamine transporter levels in the striatum. We expected the UCMS-exposed adolescents to exhibit increased tyrosine hydroxylase, and decreased dopamine transporter, in the striatum, compared to controls. Contrary to our predictions, levels of both proteins were comparable in the control and UCMS groups in both dorsal and ventral (nucleus accumbens) striatal regions [all $p>0.05$] (Table 2.6). Importantly, lack of group differences was not due to our Western blotting protocol or antibodies used (Appendix Figure A.1).

Table 2.6. UCMS-induced hyperactivity in adolescents is not accompanied by changes in striatal tyrosine hydroxylase or dopamine transporter levels.

UCMS (n = 8) and control (Con; n = 8) brains were analyzed via Western blot for tyrosine hydroxylase (TH) and the dopamine transporter (DAT) in the nucleus accumbens (NAc) and dorsal striatum (DStr). Each protein's immunoreactivity was corrected for total protein loading (LDH) immunoreactivity. Values expressed are group means ±SEMs of ratios of [TH / LDH] or [DAT / LDH], normalized to control values, for each region.

Protein	Brain Region	Con	UCMS
TH/LDH	**NAc**	1.0 (0.06)	1.0 (0.04)
	DStr	1.0 (0.05)	1.1 (0.06)
DAT/LDH	**NAc**	1.0 (0.06)	1.0 (0.06)
	DStr	1.0 (0.09)	1.1 (0.09)

Interestingly, striatal ERK2 activation also has been implicated in regard to regulation of locomotor activity (Beaulieu et al., 2006; Gainetdinov, 1999). Psychostimulant administration not only causes an increase in activity, but it also causes an increase in ERK2 activation in the striatum (Beaulieu et al., 2006; Valjent et al., 2000; Zhang, 2004). In addition to increased locomotion, dopamine transporter knockout mice also exhibit increased ERK2 activation in the striatum under basal conditions (Beaulieu et al., 2006). Psychostimulant administration in dopamine transporter knockout mice causes a paradoxical *decrease* in their locomotor activity, but this is also accompanied by inhibition of ERK2 activation in these animals (Beaulieu et al., 2006; Gainetdinov, 1999). Direct inhibition of the ERK1/2 signaling cascade by administration of a MEK inhibitor causes similar reductions in hyperactivity in normal animals (Beaulieu et al., 2006). These findings, together with our lab's own findings of increased nucleus accumbens ERK2 activation after exposure to incentive-motivational events, (Appendix B) (Kirschmann et al., 2014; Remus & Thiels, 2013; Shiflett et al., 2008), warranted an investigation of ERK1/2 activation in the dorsal and ventral (nucleus accumbens) striatum of UCMS and control groups. We found a significant increase in ERK1/2 activation at BASELINE in UCMS-exposed adolescents in the dorsal striatum [W = 26, p=0.014] (Figure 2.11A and 2.11C). There was also a significant increase in ERK1/2 activation in the nucleus accumbens of UCMS-exposed adolescents given an acute FST [W = 25, p=0.029] (Figure 2.11B and 2.11C). Thus, signaling cascades in the dorsal striatum implicated in locomotor hyperactivity are more active in UCMS-exposed adolescent rats; this provides a neurobiological correlate of the hyperactivity we observed in the UCMS group. Additionally, increased activation of signaling cascades implicated in incentive-motivation in the nucleus accumbens could imply that the UCMS-exposed adolescents found the FST to be more salient than did controls.

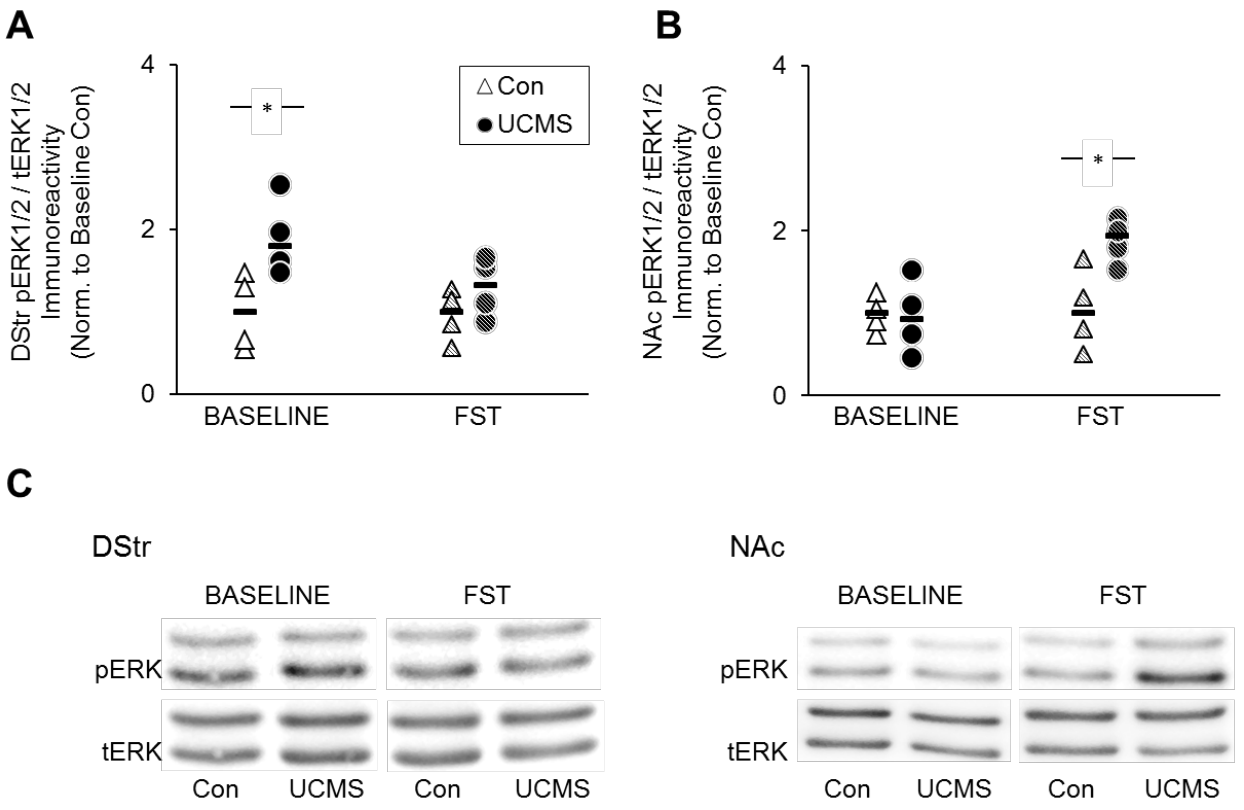


Figure 2.11. UCMS during adolescence differentially alters ERK activation in the striatum.

Striatal ERK1/2 activation [pERK1/2 immunoreactivity / tERK1/2 immunoreactivity] levels were examined via Western blot of tissue collected from UCMS (n = 8; circles) and control (Con; n = 8; triangles) groups after the last behavioral procedure. A) At BASELINE, UCMS adolescents (n = 4) had significantly higher ERK1/2 activation in the dorsal striatum (DStr) compared to Con (n = 4). B) After acute FST stress, UCMS adolescents (n = 4) had significantly higher ERK1/2 activation in the nucleus accumbens (NAc). Individual rats' data are plotted as symbols for BASELINE (solid symbols) and FST (hashed symbols) groups and are normalized to BASELINE control values; horizontal bars indicate group median values. *$p<0.05$ for between-group comparisons. C) Example blots of phosphorylated p44 (pERK1; upper bands) / p42 (pERK2; lower bands) [pERK1/2], and total p44 (tERK1; upper bands) / p42 (tERK2; lower bands) [tERK1/2] are shown for the DStr (left) and nucleus accumbens (right).

2.4 DISCUSSION

Much research has demonstrated the effects of stress to be exacerbated when exposure occurs during perinatal periods of pronounced brain maturation, i.e., postnatal day (p)1-14 [reviewed in: (Charil et al., 2010; McEwen, 2008)]. Adolescence, i.e., p28-60 now is recognized as an additional time of neurodevelopment that warrants future study. We wanted to understand the immediate effects of unpredictable chronic mild stress (UCMS) exposure during adolescence on adolescent male rats. Based on the substantial literature of UCMS exposure in adult rodents, and on the premise that adolescence is a period of vulnerability, we predicted that UCMS exposure during adolescence would cause an adolescent to exhibit depressive- and anxiety-like behaviors, and dysregulated hypothalamic-pituitary-adrenal (HPA) axis function, and that these outcomes may be more pronounced in adolescents than in adults.

Instead, we found a distinct behavioral and neuroendocrine profile in adolescent rats that differed in significant ways from our prediction. Adolescent rats exposed to UCMS during adolescence exhibited increased exploration, hyperactivity, lowered anxiety, and an absence of depressive-like behaviors. Importantly, we did see a decrease in weight gain after UCMS, similar to what was reported in adult rodents exposed to UCMS (Duncko et al., 2001; Guidotti et al., 2012; Konkle et al., 2003; Willner et al., 1996). This decrease in weight gain in adolescents exposed to UCMS was not correlated with the observed hyperactivity (Appendix Table A.1), indicating that the UCMS-induced decrease in weight gain was not simply due to increased activity. The differential effect of UCMS on weight gain and behavioral output suggests that not all systems have equivalent developmental flexibility at the time of UCMS exposure, and it implies that the weight loss typically seen after UCMS exposure cannot account for the other behavioral consequences of UCMS [also see (Flak et al., 2011)].

Although they had lower baseline levels of circulating corticosterone and lower absolute values of evoked corticosterone levels compared to controls, adolescents exposed to UCMS appeared to have intact HPA axis function. We analyzed levels of stress-, emotion-, and activity-related proteins in limbic circuitry and the striatum, and found evidence for blunting of the response to an acute stressor in the paraventricular nucleus, and increased striatal ERK activation that corresponded to the behavioral hyperactivity and a salient event (forced swim test). Together, our data suggest that: 1) UCMS during adolescence may induce a resilient phenotype that provides protection against future stress, that might be a function of stressor intensity; and 2) alternatively, UCMS during adolescence may result in detrimental changes that induce hyperactivity and increase "risky" behaviors.

2.4.1 UCMS during adolescence does not cause depressive- or anxiety-like phenotypes in adolescents

UCMS exposure has yielded depressive- and anxiety-like phenotypes in adult rodents at behavioral, neuroendocrine, and neurobiological levels (Grippo et al., 2005a; 2005b; Willner, 1997a, 2005). Here, we showed a different effect of UCMS in adolescent rodents, with a separation in the effects on depressive- versus anxiety-like behavior. Adolescent rats exposed to UCMS showed no persistent signs of depressive-like behaviors, aside from a transient reduction in sucrose preference after 1 week of UCMS that quickly recovered. Additionally, there was a robust *reduction* in anxiety-like behaviors in adolescent rats.

UCMS-exposed adolescents moved more and showed decreased anxiety in both the Open Field test and the Elevated Plus Maze. However, a third test commonly used to examine anxiety-like behaviors, the Novelty-Suppressed Feeding test, yielded no group differences. The Novelty-

Suppressed Feeding test examines a rodent's ability to solve a conflict (hunger versus exploration of an anxiogenic environment) (Bodnoff et al., 1989). Both depression and anxiety can impact an organism's conflict resolution (Dulawa & Hen, 2005; Francis-Oliveira et al., 2013). Consistent with its use as a measure of depression, performance on the Novelty-Suppressed Feeding test in adult rats was shown to be sensitive to antidepressant treatment (Bessa, 2009; Dulawa & Hen, 2005). Thus, although performance of UCMS-exposed adolescents on the Novelty-Suppressed Feeding test did not align with our other indices of *anxiety-like* behaviors (performance on the Elevated Plus Maze; center time and rearing in the Open Field), the lack of a UCMS effect on performance in the Novelty-Suppressed Feeding test suggests that UCMS-exposed adolescents had intact conflict resolution. In that sense, findings from the Novelty-Suppressed Feeding test complement observations of lower immobility in the forced swim test.

In addition to differences in behavior, there were also distinct neuroendocrine and neural profiles in our UCMS-exposed adolescents, compared to what has been reported in adults. Adult rodents exposed to chronic stress exhibit increased basal circulating levels of corticosterone, and impaired negative feedback of the HPA axis, as indicated by increased levels of circulating corticosterone after administration of dexamethasone (Grippo et al., 2005b; Helmreich et al., 2008). Dysregulated HPA axis function also has been reported in some depressed humans [(Burke et al., 2005; Heim et al., 2008); reviewed in: (Sickmann et al., 2014)]. Adult rats exhibiting the key symptom of depression, anhedonia, have also been shown to have altered levels of CREB phosphorylation in the nucleus accumbens and hippocampus (Muschamp et al., 2011; Nestler et al., 2002; Russo & Nestler, 2013). Here, we saw blunted basal corticosterone levels in adolescent rats exposed to UCMS, but no changes in phosphorylated CREB levels in

either the nucleus accumbens or the hippocampus. Thus, UCMS as we applied here does not induce a *depressive-like* behavioral, neuroendocrine, or neural phenotype in adolescent rats.

2.4.2 Does UCMS during adolescence cause resilience?

UCMS during adolescence caused a behavioral phenotype of increased exploration and decreased anxiety, accompanied by lower levels of circulating corticosterone, both at baseline and after an acute stressor. We observed no changes in the stress circuitry that are commonly seen after chronic stress: no change in glucocorticoid receptor levels or basal levels of phosphorylated CREB in the paraventricular nucleus, and no shift in basal or evoked ERK activation in the limbic system. In fact, after an acute forced swim stress (FST), phosphorylated CREB levels in the paraventricular nucleus were significantly *lower* in UCMS-exposed adolescents compared to controls, corresponding with the observed lower levels of corticosterone. Phosphorylated CREB has been implicated as a necessary co-factor for corticotrophin-releasing hormone transcription (Liu et al., 2011; Seasholtz et al., 1988), and corticotrophin-releasing hormone is one of the main signals evoked in the HPA axis response to stress [reviewed in: (Herman, 2013; Sapolsky et al., 2000)]. The fact that an acute stress (forced swim test) did not evoke signals related to a stress response could implicate resiliency in adolescents exposed to UCMS.

Decreased motivation is another core symptom of depression (American Psychiatric Association, 2013). Chronic stress has been shown to reduce hippocampal ERK in conjunction with inducing both anhedonia and decreased motivation in adult rodents (Jeon et al., 2012; Qi et al., 2008; 2006). We complemented our behavioral findings of a lack of depressive-like behavior in UCMS-exposed adolescents by confirming no change in hippocampal ERK activation.

Additionally, we have shown increases in striatal ERK to be important for appetitive cue modulation of reward-seeking, i.e. increased motivation (Remus & Thiels, 2013; Shiflett et al., 2008). Thus, the UCMS-exposed adolescents did not appear to show signs of decreased motivation, though this remains to be confirmed with a more direct assay of motivated responding (e.g., a progressive ratio test for food reward). We found *increased* ERK activation in the nucleus accumbens after an acute stressor (i.e., the FST) in UCMS-exposed adolescents. Based on our lab's work on the role of striatal ERK activation in response to salient cues [(Appendix B), (Kirschmann et al., 2014; Remus & Thiels, 2013; Shiflett et al., 2008)], one interpretation of this would suggest that the UCMS group found the FST to be more salient than controls.

Our findings do align with the few other studies that utilized more aversive stressors during early adolescence and examined effects *in adolescence*. Mice exposed to chronic restraint stress (7 days) in early adolescence exhibited hyperactivity and decreased anxiety in the Open Field test (Ito et al., 2010), and rats and mice exposed to stressors including restraint, forced swim, shaker, and platform stress in early adolescence showed decreased anxiety-like behaviors in the Open Field test and the Elevated Plus Maze (Jacobson-Pick & Richter-Levin, 2010; Peleg-Raibstein & Feldon, 2011). We believe that the UCMS paradigm is a better model of naturalistic stress, as opposed to the more severe stresses used in the aforementioned studies, and it encompasses the entirety of adolescence, as opposed to only the early portion. Our work also corresponds to findings from studies utilizing social isolation as a stressor in adolescence. Hyperactivity in an open field, lower levels of corticosterone after an acute stressor and at baseline, and a lack of behavioral despair in the FST have all been reported in studies of adolescent rats exposed to social isolation throughout adolescence, although some results tend to

be more pronounced in females (Adzic et al., 2009; Douglas et al., 2004; Hong et al., 2012; Levine et al., 2007; Weintraub et al., 2010). Thus, a variety of stress exposure paradigms, including our more naturalistic and mild, as opposed to very severe, UCMS stressors, induce low anxiety in adolescent rats.

Another possible interpretation of the observed behavioral and neuroendocrine changes after UCMS during adolescence is that the daily occurrence of unpredictable stressors may actually be mildly stimulating. In some cases, it has been suggested that mild stressors applied to a rat while socially isolated can actually serve as a form of environmental enrichment (L.P. Spear, personal communication). Additionally, one group has shown that the mood and anxiety changes observed (in adulthood) after brief juvenile stress exposure were reversed after subsequent environmental enrichment from adolescence through adulthood (Baune et al., 2009b). Further work aimed to parse apart whether the stressors in this UCMS protocol might be mildly rewarding could help discern if enrichment is occurring.

2.4.3 Is UCMS in adolescence detrimental?

An alternative possibility to UCMS exposure during adolescence causing resilience in adolescent rats is that the behavioral, neuroendocrine, and neural profiles actually resemble detrimental outcomes. For example, the observed hyperactivity in UCMS-exposed adolescents is one of the key symptoms in attention-deficit hyperactivity disorder (ADHD), which also is most prevalent at this young age (American Psychiatric Association, 2013). A genetic model for ADHD consists of mice that were selectively bred for low anxiety-like behavior (based on >60% preference for entering the open arm on the Elevated Plus Maze); these mice also exhibited hyperactivity in the home cage, and in the Open Field (Bunck et al., 2009; Yen et al., 2013). Also, interestingly, a

region important for reward processing and emotionally salient events (nucleus accumbens) seemed to be more activated in the UCMS group at after an acute forced swim stress (increased ERK2 activation). This suggests that the UCMS-exposed adolescents are not only hyperactive, but they may potentially be more reactive to reward-predictive cues. We have observed elevated ERK2 signaling in the nucleus accumbens to be associated with enhanced reward-seeking (Mauna et al., in prep). Hence, increased ERK2 activation in the nucleus accumbens is more consistent with a profile of hyper-motivated state

2.4.4 Future Directions

Now that we have provided critical information on the immediate effects of UCMS during adolescence, *in adolescent rats*, a number of exciting follow-up studies exist. Importantly, in order to better distinguish whether UCMS during adolescence yields a resilient or an impaired adolescent, additional work is needed. First, it would be instructive to assay for impulsive-like behaviors in late adolescent animals after UCMS exposure. If adolescents demonstrated signs of increased impulsivity on tests such as the five-choice serial reaction time task or delayed discounting, this would provide support for the interpretation that UCMS during adolescence is detrimental. More importantly, it is imperative to allow a group of adolescents that were exposed to UCMS during adolescence to age to adulthood, and re-examine behavioral, neuroendocrine, and neural changes at baseline, and in response to acute and chronic stress exposure. We predict that, if UCMS exposure during adolescence does result in beneficial behavioral, neuroendocrine, and neural changes, subsequent stress exposures would continue to produce less depressive- and anxiety-like behaviors, and evoke less neuroendocrine and neural stress responses. However, if in fact the observed changes induce more of an ADHD-like state in the adolescents, clinical

evidence suggests that additional stress in adulthood can precipitate anxiety disorders (Adler et al., 2004; Fones et al., 2000). It also would be pertinent to replicate the current study using female rats. We acknowledge that there are sex differences in anxiety- and depressive-disorders, with women at greater risk than men (Nestler et al., 2002), and this disparity is also seen in adolescent girls (Angold & Worthman, 1993). Additionally there are clear sex differences in the effects of stress [e.g.: (Sjöberg et al., 2005; Wood & Shors, 1998)], and some studies of chronic stress in adolescence have demonstrated more pronounced effects in females compared to males [e.g.: (Bourke & Neigh, 2011; McCormick & Mathews, 2007)]. Together, these additional data would greatly strengthen our current interpretations, and could provide useful comparisons between sexes, and late-adolescent brain circuitry functioning and later adult profiles.

2.4.5 Conclusions

Here, we show a robust hyperactive, low-anxiety phenotype in adolescent male rats exposed to UCMS during adolescence. Exposure to an additional acute stressor in these rats yielded less of a stress response in neuroendocrine and brain stress systems, compared to controls. Additionally, UCMS-induced hyperactivity in adolescents corresponded with increased ERK activation in the dorsal striatum. The behavioral and neuroendocrine changes we observed in adolescent rats are in contrast to what has been established in the literature for adult rats exposed to UCMS. It could be that, because adolescence is a time of major neurodevelopment, behavioral, neuroendocrine and neural systems are able to compensate for stress-induced perturbations in normal functioning. Thus, adolescent animals exposed to chronic mild stress during this time may actually develop resiliency towards additional stressors. Alternatively, there may a negative shift of systems during adolescence, resulting in an impaired, hyperactive

phenotype that is detrimental. Our work has provided an important missing piece of data for the field, in understanding how UCMS in adolescence affects adolescent behavior and neuroendocrine function; and it raises several important questions for future work, including whether UCMS exposure during adolescence may serve as a better model of resiliency, or of detrimental hyperactivity. Knowledge of how chronic stress during adolescence impacts the behavioral, neuroendocrine, and neural functioning *in adolescence* is critical for gaining an understanding of what may precipitate neuropsychiatric disorders, or what could prevent development of those same disorders later in life.

3.0 EARLY LIFE EXPERIENCE MODULATES THE EFFECTS OF UNPREDICTABLE CHRONIC MILD STRESS DURING ADOLESCENCE

3.1 INTRODUCTION

Exposure to stress early in life can have drastic influence on behavioral and endocrine stress responses (Levine, 2005). In humans, early life stress is a major risk factor for numerous neuropsychiatric disorders, including depression and anxiety (Heim & Nemeroff, 2001). Adolescence is an important time of neurodevelopment that may also be a time of increased vulnerability to stress (Doremus-Fitzwater et al., 2010; Lupien et al., 2009; Spear, 2000). One system that has a distinct profile of function during adolescence is the hypothalamic-pituitary-adrenal (HPA) axis (Levine, 2001; Lupien et al., 2009; Romeo et al., 2006; Vazquez, 1998). Animal models have served useful in understanding the neurobiological changes underlying the effects of early life stress. Though much is known about the long-term consequences of stress early in life on behavior and neuroendocrine function, less is known about the effects of chronic stress during adolescence *in adolescence*. The most well-validated animal model of stress-induced depression in adult rodents is unpredictable chronic mild stress (UCMS) (Willner, 1997b, 2005).

In rodents, adolescence is considered to span across puberty from postnatal day (p) 28-42, and can also include periods of early- (p21-34) and late-adolescence (p46-59) (Eiland & Romeo, 2013; Tirelli et al., 2003). Because rodent adolescence is limited to just a few weeks (McCormick & Mathews, 2007; Tirelli et al., 2003), experimental manipulations often begin close in time to weaning age (peri-weaning) (Eiland & Romeo, 2013). Unless researchers have the ability/resources to maintain their own breeding colonies of rodents, they must have their research animals shipped to their facility. During the process of shipping, rodents are likely exposed to a variety of stimuli that are often used as discrete stressors themselves: noise, vibrations, changes in temperature, and noxious odors are just a few (Laroche et al., 2009; Ogawa et al., 2007). Thus, shipping itself is stressful to rodents. One point of contention in the field of adolescent work is that of the peri-weaning experience – in particular, whether a young rat experiencing the stress of being removed from the lactating dam and then immediately shipped from a supplier to a research facility can be an appropriate animal model for any research (Laroche et al., 2009; Wiley & Evans, 2009). Wiley and Evans (2009) conducted a descriptive study in an attempt to assess whether there were different pharmacological effects of a few drugs on adolescent rats that were weaned and shipped versus animals that were born in the facility (e.g., born in-house). In that study, there were not striking qualitative differences between the two peri-weaning experiences, though the authors noted there were some quantitative differences across different doses of the pharmacological manipulations (Wiley & Evans, 2009). We wanted to test the effects of UCMS in adolescents that had a different peri-weaning experience from the standard “wean and ship” condition used in our previous study (**Chapter 2**).

Aside from the potential immediate detrimental effects of the early-life stress of weaning/shipping, another notion of concern arises from what some term the "two-hit hypothesis." Originally stemming from work in schizophrenia, this hypothesis posits that early life genetic or environmental factors interfere with development of the central nervous system, which produces a vulnerability to the psychiatric disorder (first "hit"); but the onset of symptoms does not occur until a subsequent "hit" from additional environmental factors, such as stress [reviewed in: (Maynard et al., 2001)]. In terms of the peri-weaning concern, it could be that the combination of weaning and shipping is detrimental enough to alter developmental trajectories and induce psychiatric vulnerabilities, putting adolescent animals at heightened risk, prior to any experimental manipulations at all.

Here, we examined the effects of 5 weeks of UCMS exposure in adolescent (i.e., p28-60) male rats that had been shipped with a foster dam and weaned 3 days later in-house (ship-with-dam). We evaluated behavioral and neuroendocrine consequences during the fifth week, i.e., in late adolescence. Contrary to what we previously found in wean-and-ship adolescents (**Chapter 2**), ship-with-dam adolescents exposed to UCMS during adolescence exhibited an overall similar behavioral and neuroendocrine profile compared to that of controls. Taken together with the outcome from our previous study (**Chapter 2**), these findings suggest that the environmental conditions immediately preceding weaning are critical influences on experimental outcome. However, as additional analyses discussed below show, the interpretation of our findings is complex.

3.2 MATERIALS AND METHODS

3.2.1 Animals

We defined rodent adolescence as starting one week post-weaning (postnatal day 28; p28), spanning through puberty, and ending around p60. This broad age range included the standard conservative age range (p28-42) that is anchored around puberty and behavioral changes (Spear, 2000), as well as the period that is often considered to be late adolescence (Eiland & Romeo, 2013; McCormick & Mathews, 2007; Tirelli et al., 2003). A total of fifty-eight adolescent male Sprague-Dawley rats (Charles River, Portage, MI) were used across multiple studies. All rats were housed in pairs in plastic cages (40 x 22 x 19cm), in a temperature and humidity controlled environment, with standard rat chow (Purina) and water available *ad libitum*. Rooms were on a standard 12hr light-dark cycle (lights on at 0700). The University of Pittsburgh Institutional Animal Care and Use Committee approved all procedures used.

3.2.1.1 Ship with Dam Condition

Male rats (n = 24) were shipped from the supplier on p18 with a lactating foster dam (n = 8 pups/foster dam). The supplier was asked to pull pups from as many different litters as possible, to reduce the chances of receiving littermates. Upon arrival, pups and dams were left undisturbed in the cage. Rats were removed from the foster dam on p21 and housed in pairs for the remainder of the study unless otherwise specified. Rats were again left undisturbed until behavioral testing began on p24.

3.2.1.2 Wean and Ship Condition

Rats (n = 24) were weaned and shipped from the supplier on p21. Upon arrival, rats were immediately housed in pairs, and left undisturbed until behavioral testing began on p23. Most of these rats' (n = 20) data were previously reported (**Chapter 2**) and are used as a comparison to the other groups.

3.2.1.3 In-house Controls

In order to determine which shipping style most resembled what would be found with rats born in our own facility, we conducted an additional experiment on control rats born in-house. Ten pregnant dams were delivered from the supplier (Charles River) on gestational day 14-16. Pregnancy was determined at the Charles River facility by the presence of a vaginal plug. Dams were singly-housed plastic cages, with standard rat chow (Purina) and water available *ad libitum*. Rooms were maintained on a standard 12hr light-dark cycle (lights on at 0700), in a temperature and humidity controlled environment. Dams were left undisturbed until birth of pups, with the exception of visual inspection of cages. The day of birth of the litter was recorded as p0; litter sizes ranged from 9-14 pups. Animals were culled to 9-12 pups/dam on p2-3 (equal males and females were kept when possible), and were again left undisturbed except for weekly cage changes/litter weights until weaning on p21. At weaning, 10 males were pair-housed for the remainder of the experiment and were exposed to control conditions. Only 1 male was kept from each dam for behavioral analyses to prevent litter effects (Holson & Pearce, 1992). The remaining rats were removed from the housing room.

3.2.2 Unpredictable Chronic Mild Stress (UCMS) Protocol

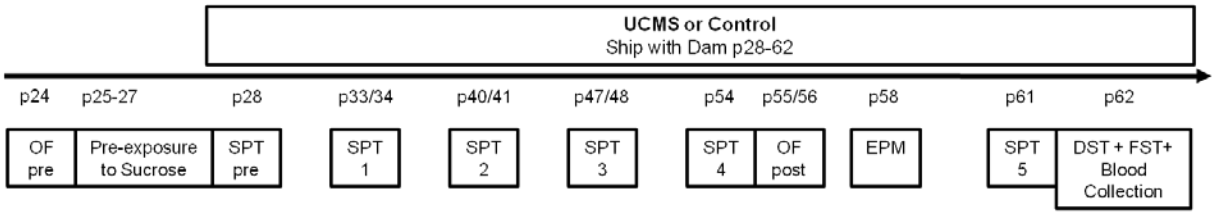
UCMS was conducted as described previously (**Chapter 2**). Briefly, baseline measurements were collected over a period of 5-9days before adolescent rats were divided into UCMS and control (Con) groups. UCMS rats were single-housed and exposed to a series of intermittent, mild stressors presented in varying order over the course of a week, for a total of five consecutive weeks (Figure 3.1A). This UCMS paradigm, modeled after Willner (1997a, 2005), was demonstrated to induce depressive-like behaviors in adult rats (Grippo et al., 2003; 2005a; 2005b; Grønli et al., 2004; Stedenfeld et al., 2011). We also saw an effect of this UCMS paradigm in wean-and-ship adolescent rats (**Chapter 2**). Figure 3.1B shows an example of a typical week during UCMS. The following individual stressors were used in the UCMS protocol: white noise (3 or 5hr; radio static, 85dB); 45-degree cage tilt (6 or 18hr); continuous overnight illumination; overnight water deprivation (16hr) followed by 1hr of empty water bottle replacement; food restriction (2hr); stroboscopic lighting (6, 10, or 18hr; Eliminator Lighting mini-strobe E105; 8–10 flashes per second, 25W); overnight paired housing with another UCMS-exposed rat (18hr); damp/dirty bedding (300–500ml lukewarm water added to cage bedding; 18hr); and predator odor exposure (10–60min exposure to 20µL undiluted 2,4,5-trimethylthiazoline [Contech Enterprises, Inc., Canada] placed onto a piece of gauze inside an open Eppendorf micro-centrifuge tube [Fisher Scientific, Pittsburgh, PA] and hung outside each cage). Control rats were pair-housed, and underwent standard animal care throughout the course of the experiment (weekly cage changes and daily weighing). Controls were single-housed overnight once a week for the sucrose preference test (described below). In-house rats (n = 10) were exposed to control conditions only; a subset of the ship-with-dam (n = 4) and wean-and-

ship (n = 4) controls were tested in parallel with the in-house group, to ensure that group differences were not due to cohort effects (Figure 3.1C).

3.2.3 Behavioral Procedures

Baseline measures were taken prior to the start of UCMS. Assessments of weight occurred daily, assessments of anhedonia (sucrose preference test) occurred weekly, and behavioral tests of anxiety- and depressive-like behaviors were conducted during the last week of the five-week UCMS protocol (Figure 3.1A). For in-house controls, behavioral tests were conducted a few days after weaning (Figure 3.1C). Rats underwent only one behavioral assessment per day. Excluding the sucrose preference test (conducted in the housing rooms overnight), each behavioral test occurred during the light cycle in a single behavioral testing room. Rats were transported in their home cages and were allowed 5-10min to habituate to the testing room before starting each test.

A



B

	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Sucrose Preference Test			5pm →	9am			
Food Deprivation		5-7pm		3-5pm			
Water Deprivation		5pm →	9am			6pm →	10am
Empty Water Bottle			9-10am				
Overnight Illumination	7am			7pm →	7am		7pm →
Cage Tilt	3:30pm →	9:30am			10am-4pm		
Paired Housing					4pm →	10am	
Damp Bedding					4pm →	10am	
Intermittent White Noise				4-9pm			12-3am
Stroboscopic Lighting		1-7am				8pm →	6am
Predator Odor	2:30-3pm						

C

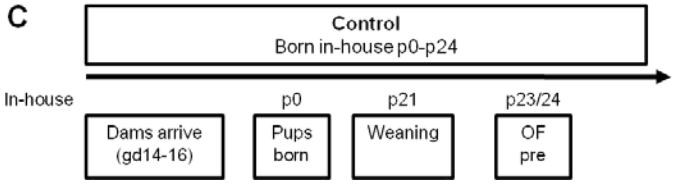


Figure 3.1. Experimental details.

A) Experimental Timeline for ship-with-dam groups. Age in postnatal days (p) for each test is indicated. Young rats were shipped from the supplier with a foster dam on p18; rats were weaned and pair-housed on p21. B) Example of a typical week in the UCMS protocol. Rats were single-housed, and were exposed to 2-3 stressors each day.

C) Experimental Timeline for in-house group. Untimed pregnant dams were shipped from the supplier and were left undisturbed until birth of litter, marked as p0. Litters were culled on p2-3 and left undisturbed until litters were weaned and male rats were pair-housed on p21. UCMS: Unpredictable Chronic Mild Stress; OF: Open Field Test; SPT: Sucrose Preference Test; EPM: Elevated Plus Maze; DST: Dexamethasone Suppression Test; FST: Forced Swim Test; gd: Gestational day.

3.2.3.1 Open Field Test

The Open Field measures anxiety-like and exploratory behavior in rodents (Brooks & Dunnett, 2009; Choleris et al., 2001; Coelho et al., 2014; Schmitt & Hiemke, 1998). It can also be used in a longer protocol (1-2hr exposure) to assess individual differences in stress response by measuring locomotor activity in response to a novel environment (Calvo et al., 2011; Jama et al., 2008; Kabbaj et al., 2000; Stead et al., 2006; Stedenfeld et al., 2011). Rats were placed into an open chamber (43 x 43cm; MedAssociates, St. Albans, VT) under dim lighting (<10lux). Activity in the horizontal and vertical planes was monitored for two hours, via horizontal and vertical infared beam breaks across 16 evenly-spaced squares. Rats were given a pre-test (pre-stress) on p23/24 (early adolescence) before assignment into UCMS or Con group. Rats were tested again after ~4 weeks of UCMS or control conditions (post-stress) on p55/56 (late adolescence). Because rats were tested multiple times in the Open Field and novelty of the environment is inherent in the test, visual, tactile, and olfactory cues were altered between each Open Field exposure. The total distance traveled during the two hours provided an index of overall locomotor activity. Additional measures, such as time spent in the periphery of the arena, time spent in the center, and amount of rearing were examined to provide an index of anxiety-like behavior (Schmidt & Duman, 2007).

3.2.3.2 Sucrose Preference Test

Rats were pre-exposed to having two drinking bottles in the home cage; one bottle had regular tap water and the other had sucrose solution (2% wt/vol; Fisher Scientific) from p25-p27. Next, baseline sucrose preference scores were taken: all rats were single-housed overnight, with free access to two bottles placed in the center of the cage lid (one with regular tap water and one with 2% sucrose solution). Bottles were weighed pre- and post-test. Sucrose preference was quantified

as the ratio of total sucrose intake (g) to total fluid intake (g) x 100. The morning after the baseline sucrose preference test had completed (p28), rats were returned to paired housing. Rats then were randomly assigned to the control and UCMS groups, with the restrictions that the average baseline sucrose preference and average locomotor activity scores during the pre-stress Open Field test were comparable between the two groups. UCMS rats then were single-housed in another room for the remainder of the study. Sucrose preference tests occurred once a week, with control rats being shifted to single-housing one night per week during each sucrose preference test. Bottle position was counterbalanced across rats/weeks to avoid potential side biases.

3.2.3.3 Elevated Plus Maze

Rats were tested on the Elevated Plus Maze under dim lighting (open arms 5-10lux; closed arms <2lux) after 4 weeks of UCMS (p58). Rats were placed, facing a closed arm, in the 10 x 10cm central area of a plus-shaped maze that was raised 83cm above the ground. Two of the four arms (50 x 10cm) were open and two were closed. The rats' exploratory activity was recorded for 5min and scored offline. Cumulative time and entries into open and closed arms were recorded, along with the latency to the first entry into an open arm (Walf & Frye, 2007).

3.2.4 HPA Axis Analyses

3.2.4.1 Baseline Corticosterone

On the final day of the experiment (p62), some rats (n = 3 UCMS, 3 Con) received a single subcutaneous injection of 150mM NaCl (saline; 0.3ml) 6hr prior to sacrifice. Immediately before decapitation, rats were transported in their home cage to the behavioral testing room, and were

anesthetized with an intraperitoneal injection of chloral hydrate (300mg/kg; Sigma-Aldrich, St. Louis, MO; dissolved in saline). Brains and blood were then collected (described below).

3.2.4.2 Dexamethasone Suppression Test and Forced Swim Stress

The Dexamethasone Suppression Test examines for dysregulated HPA axis function: dexamethasone, a synthetic glucocorticoid agonist, is administered in order to suppress corticosterone release via negative feedback mechanisms (Helmreich et al., 2008). The forced swim test (FST) is a stress-based test that is sensitive to the behavioral effects of antidepressants (Castagné et al., 2011; 2009; Porsolt et al., 1978; Schmidt & Duman, 2007); here we took advantage of the FST as an acute stressor. On the final day of the experiment (p62), some rats received a single subcutaneous injection of either dexamethasone (DEX; 100µg/ml/1kg; University of Pittsburgh pharmacy; n = 3 UCMS, 3 Con) or vehicle (saline; 0.3ml; n = 3 UCMS, 3 Con), approximately 6hr prior to sacrifice. Immediately before decapitation, rats were transported to the behavioral testing room in their home cage, and were given a 5min FST in a clear Plexiglas cylinder (~35cm high, 19in internal diameter; water ~20°C). Behavior was recorded and scored offline for latency to first immobile episode and total immobility time; immobility was defined as the absence of any movements except to keep nose above water for ≥1s. At the conclusion of the swim stress, rats were anesthetized, and brains and blood were collected (described below). The timing and dosage of DEX injection were based on published time-course and dose-response studies (Helmreich et al., 2008; Lurie et al., 1989; Oxenkrug et al., 1984).

3.2.5 Tissue and Plasma Collection

On the final day of the experiment (p62), rats were anesthetized with an intraperitoneal injection of chloral hydrate between 4-6pm. Rats were decapitated; brains were rapidly collected and flash frozen in isopentane (Acros Organics, Morris Plains, NJ), and were stored at -80°C for tissue analyses (related to other ongoing experiments). Trunk blood was also collected into ice-cold, heparin-coated tubes (BD Vacutainer 6ml tubes, sodium heparin 95USP units; Becton, Dickinson and Co., Franklin Lakes, NJ) at time of decapitation, and then centrifuged at 3,000rpm for 20min at 4°C; plasma was collected and stored at -80°C until later analyses (see ELISA Analyses, below).

3.2.6 ELISA Analyses

As described above, plasma was collected and stored at -80°C until analysis of circulating corticosterone levels. Commercially-available ELISA kits were used, according to manufacturer's protocols, to assay for corticosterone (Immunodiagnostic Systems, Scottsdale, AZ). Coefficients of variation ranged from 0.01-0.20; average intra-assay coefficient of variation was 0.12; and assay sensitivity was 550pg/ml.

3.2.7 Statistical Analyses

Four rats of the original 58 (2 ship-with-dam, 1 wean-and-ship, 1 in-house) were excluded from analyses because of extraordinarily high initial locomotor activity in the initial Open Field test (>2std above mean). Final group sizes were 35 controls and 19 UCMS. For UCMS assessments

group sizes were: UCMS (n = 9 ship-with-dam, 10 wean-and-ship), Control (n = 9 ship-with-dam, 10 wean-and-ship). For analyses with in-house rats group sizes were: Control (n = 13 ship-with-dam, 13 wean-and-ship, 9 in-house). For analyses of data collected throughout the UCMS exposure (weight, sucrose preference), scores were compared using repeated measures analyses of variance (ANOVAs) with group (UCMS, Con) and shipping style (ship-with-dam, wean-and-ship) as between-subject factors and day as the within-subject factor, followed by post-hoc pairwise comparisons using t-tests with Bonferroni's correction where necessary. For end-point analyses, ANOVAs with group (UCMS, Con) and shipping style (ship-with-dam, wean-and-ship, in-house) as the between-subjects factors were conducted. Latency data were normally distributed unless otherwise indicated; when necessary, data were transformed ($\log_{10}$) prior to statistical analyses. For ELISA analyses, group sizes fell below n = 5, and nonparametric tests (Wilcoxon Rank-Sum) were conducted with group (UCMS, Con), shipping style (ship-with-dam, wean-and-ship), and end condition (BASELINE, FST) as between-subjects factors. Statistical analyses were performed using the SPSS software package version 20.0 (SPSS, Chicago, IL). Significance level was set to $\alpha \leq 0.05$.

3.3 RESULTS

3.3.1 UCMS during adolescence does not alter weight gain or cause depressive-like behavior in ship-with-dam adolescent rats

While investigating the effects of UCMS during adolescence in adolescent male rats, we also wanted to examine whether peri-weaning experience (i.e., shipping conditions) had an impact on the effects of chronic stress in adolescence. Here, we first present the effects of UCMS during adolescence in adolescent rats that were shipped with a foster dam and weaned in-house and therefore had the stress of shipping separated in time from the stress of being weaned (ship-with-dam condition). Because almost all of the UCMS effects were absent in ship-with-dam rats, we then compare these data to data obtained from the same experimental protocol, using animals that experienced weaning and shipping stress concurrently (wean-and-ship condition; see **Chapter 2**), and discuss significant interactions between groups with different early life experiences. Finally, because our analyses indicated that key differences in our findings may lie between the *control groups*, we examine the rats in the aforementioned peri-weaning conditions (ship-with-dam, wean-and-ship) with rats that were born in our own facility and experienced no peri-weaning shipping stress at all (in-house controls).

To determine the behavioral effects of UCMS during adolescence in ship-with-dam rats, we monitored weight gain and sucrose preference during 5 weeks of UCMS exposure, and we performed behavioral assays during the fifth week of UCMS in late-adolescent rats. For reporting purposes, all behavioral results collected during the fifth week of UCMS will be referred to as “post-stress.” A key feature of UCMS in adult male rodents is a substantial blunting of weight gain over time (Duncko et al., 2001; Konkle et al., 2003; Murison & Hansen,

2001; Willner et al., 1996). We saw a similar reduction in weight gain in wean-and-ship male adolescent rats (**Chapter 2**). However, we saw no such reduction in weight gain after UCMS exposure during adolescence in ship-with-dam adolescents. Figure 3.2A shows group average weights across the course of the experiment. Weights increased over time in both groups [main effect of day: $F(9,144) = 1331.46$, $p<0.001$], but there were no differences between Con and UCMS groups [no day x group interaction: $F(9,144) = 1.61$, $p>0.05$]. UCMS did not reduce total weight gain, either in absolute grams [$t(16) = 1.25$, $p>0.05$] (Figure 3.2B), or in percentage change from baseline [$t(16) = 1.60$, $p>0.05$] (data not shown).

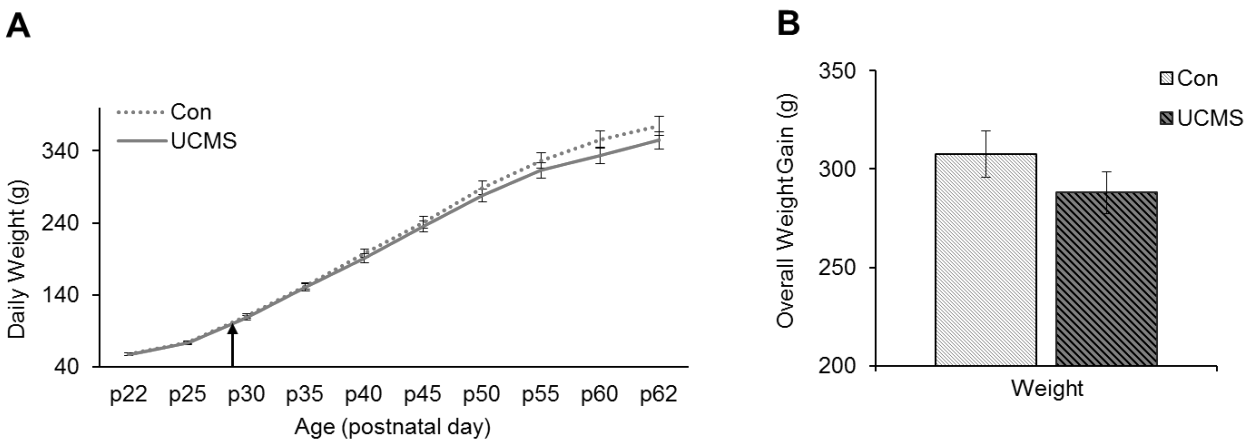


Figure 3.2. UCMS during adolescence does not affect weight gain in ship-with-dam rats.

A) Daily weights in grams (g) throughout adolescence for ship-with-dam rats in the UCMS group (n = 9) and the control group (Con; n = 9). All rats gained weight during adolescence, and this was not affected by UCMS. Arrow indicates start of UCMS exposure. B) Total weight gained after 5 weeks of UCMS or control conditions. Adolescent ship-with-dam rats exposed to UCMS gained as much weight as did controls. Overall weight gain (g) was calculated as [final weight – baseline weight]. Values show group means ± SEMs.

Next, we examined whether there were depressive-like behavioral consequences in ship-with-dam adolescent rats exposed to UCMS during adolescence, by measuring sucrose preference over time. Adolescent controls preferred sucrose over regular water (≥70-80%) each week (Figure 3.3A). These data fall in line with adult literature (Grippo et al., 2003; 2005a; 2005b; Stedenfeld et al., 2011), and align with our findings in wean-and-ship control adolescent rats (**Chapter 2**). We saw a blunted effect of UCMS on sucrose preference in ship-with-dam adolescents, compared to what we observed in wean-and-ship adolescents. Similar to controls, UCMS rats preferred sucrose over water to a large degree each week. Although sucrose preference increased over time [main effect of day: $F(5,80) = 6.41$, $p<0.001$], there were no group differences between control and UCMS groups [no day x group interaction: $F(5,80) = 1.73$, $p>0.05$]. Compared to the group's own baseline, UCMS rats did have a reduction in sucrose preference after one week of UCMS [paired t-test: $t(8) = 2.49$, $p=0.037$] (Figure 3.3B); however, UCMS preference scores still did not differ significantly from controls [$t(16) = 0.69$, $p>0.05$]. Together, these data suggest that UCMS during adolescence does not impair weight gain or cause persistent anhedonia behavior in ship-with-dam adolescent rats. The lack of an effect of UCMS on weight gain in ship-with-dam rats was contrary to the significant reduction in weight gain observed in wean-and-ship rats (**Chapter 2**). The transient anhedonia after 1 week of UCMS in ship-with-dam rats was similar to our observed UCMS effects in wean-and-ship rats (**Chapter 2**), but was not as robust: ship-with-dam UCMS rats only significantly reduced preference compared to their own baseline and did not differ significantly from ship-with-dam control levels.

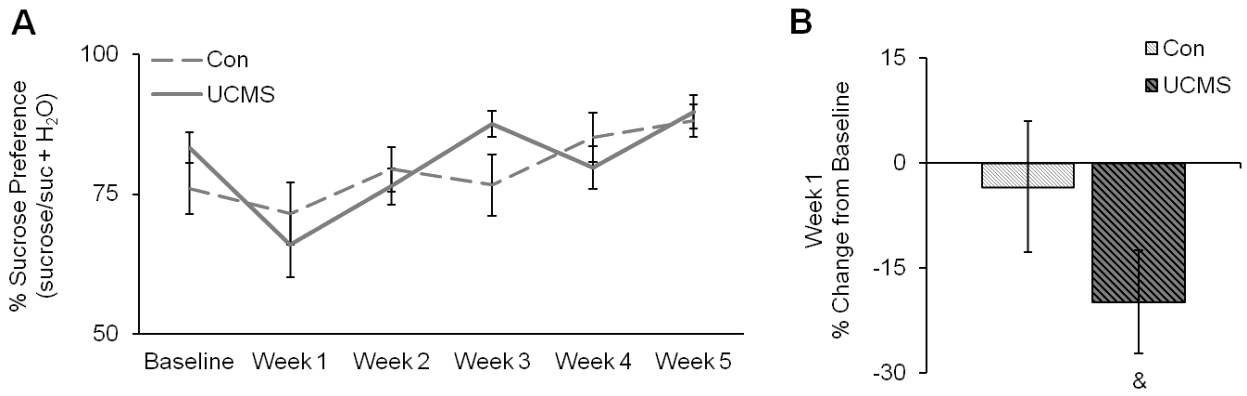


Figure 3.3. UCMS during adolescence does not cause anhedonia in ship-with-dam rats.

A) Weekly sucrose preference scores throughout adolescence for ship-with-dam rats in the UCMS group (n = 9) and the control group (Con; n = 9). Bottles of 2% sucrose and tap water were weighed pre- and post-test, and sucrose preference was quantified as [total sucrose intake (g) / total fluid intake (g)] x 100. Both groups preferred to drink sucrose. B) Week 1 percent change in sucrose preference was calculated for each group as [(week 1 preference – baseline preference) / baseline preference] x 100. One week of UCMS did cause a significant reduction in sucrose preference compared to the group's own baseline, but UCMS values did not differ from Con, and the effect was not sustained. Values show group means ± SEMs. & $p \leq 0.05$ for within-group comparisons to baseline.

3.3.2 UCMS during adolescence causes hyperactivity, but does not alter anxiety-like behaviors in ship-with-dam adolescent rats

We next assessed exploration and anxiety-like behaviors after adolescent UCMS exposure in ship-with-dam adolescent rats with the Open Field and Elevated Plus Maze. Figure 3.4A shows locomotor activity, i.e., total distance traveled in a 2hr Open Field test before UCMS began (pre-stress) and during week 5 of UCMS (post-stress). There was a main effect for test [$F(1,16) = 109.72$, $p<0.001$], and group [$F(1,16) = 192.59$, $p<0.001$]. Both groups increased total ambulatory distance in the Open Field post-stress, compared to the Open Field pre-stress. There was a significant group x test interaction [$F(1,16) = 14.39$, $p=0.002$]: although adolescent rats from the two groups displayed similar exploratory locomotor activity pre-stress, UCMS-exposed adolescents moved more over 2hr in the post-stress Open Field than did controls [post-hoc t-test: $p=0.002$]. Additionally, the percent increase in distance traveled from pre- to post-stress Open Field was significantly greater for UCMS rats compared to controls [$t(16) = 2.42$, $p=0.028$]. Figure 3.4B shows locomotor activity in the post-stress Open Field after 15min. UCMS caused an increase in ambulatory distance that was evident within the first 15min [$t(16) = 2.45$, $p=0.026$]; the higher level of activity was then maintained throughout the 2hr test (as described above). Figure 3.4C shows vertical time (rearing) in the pre- and post-stress Open Field tests. Rearing results showed a different pattern compared to that observed for ambulatory distance: there was a main effect for test [$F(1,16) = 31.37$, $p<0.001$], but only a trend for group [$F(1,16) = 3.60$, $p=0.076$]. Both groups increased rearing in the post-stress Open Field compared to pre-stress Open Field, regardless of stress condition [no significant group x test interaction: $F(1,16) = 0.977$, $p>0.05$]. There were also no group differences in rearing after 15min in the post-stress Open Field [$t(16) = 0.93$), $p>0.05$] (Figure 3.4D). When examining the total amount of time

spent in the center of the Open Field arena, UCMS-exposed adolescent rats spent more time in the center than controls during the full 2hr [$t(16) = 3.10$, $p=0.007$], a difference that developed over the course of the 2hr test, but was not evident during the first 15min [$t(16) = 1.76$, $p>0.05$] (Figure 3.4E). Similar to what we observed in wean-and-ship adolescent rats (**Chapter 2**), we found that, despite spending more absolute time in the center compared to controls, the relative amount of time that UCMS-exposed ship-with-dam adolescent rats allocated to various behaviors was very similar to the relative distribution exhibited by the ship-with-dam control group (Figure 3.4F). Both groups spent a majority of the time in the center resting (~45% for each), and divided the remaining time in the center fairly evenly between fine motor movements (stereotypic behaviors), rearing, and ambulation [repeated measures ANOVA: no main effect of group or group x behavior interaction, both $p>0.05$]. Together, performance in the Open Field suggests an upward shift in overall exploratory activity for UCMS-exposed ship-with-dam adolescent rats. These results are consistent with, but not as robust as, our findings in wean-and-ship adolescent rats (**Chapter 2**).

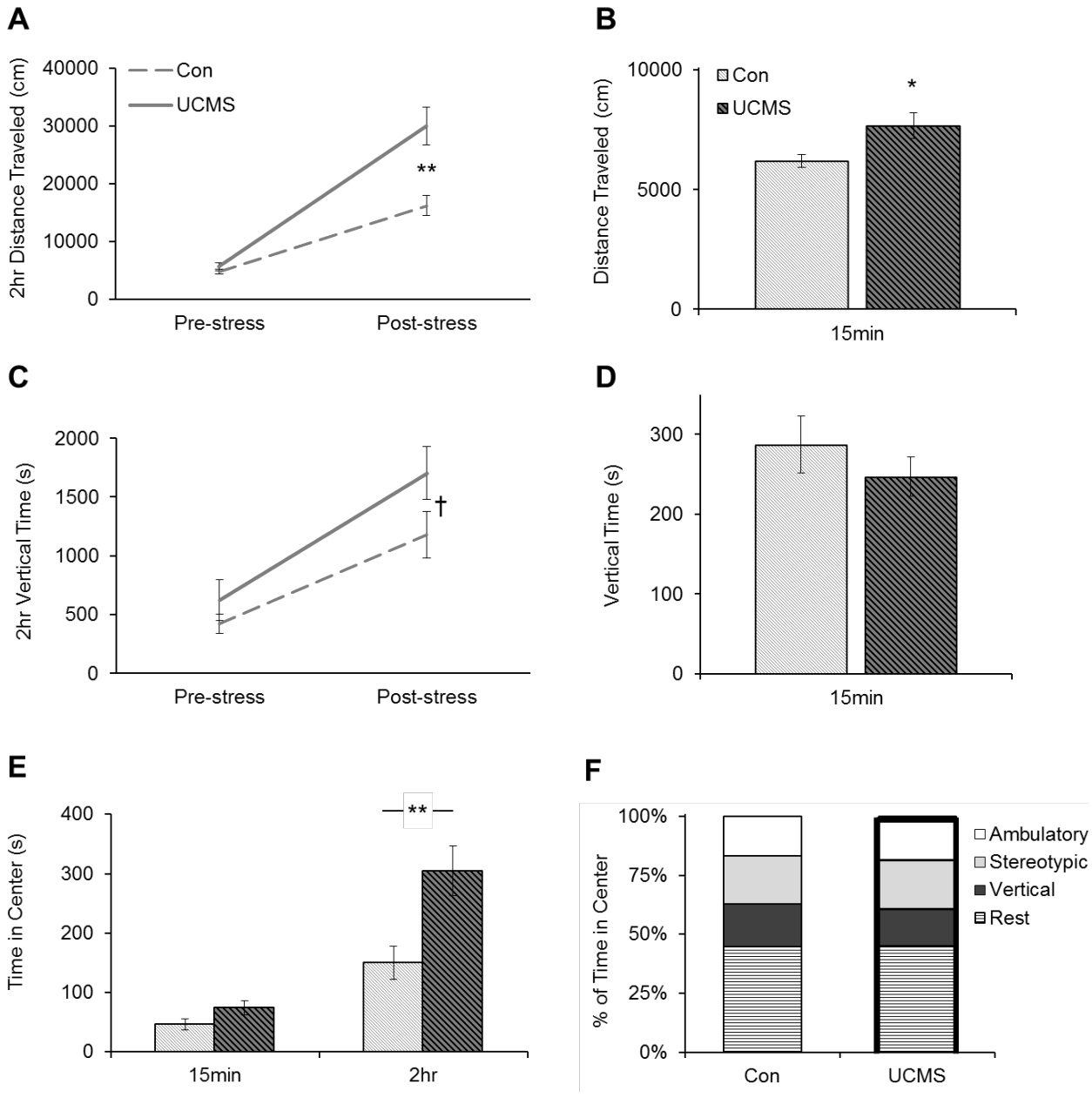


Figure 3.4. UCMS during adolescence causes hyperactivity and increased exploration in the Open Field in ship-with-dam rats.

A) Open Field tests were given prior to the start of UCMS (Pre-stress) and during week 5 of UCMS during adolescence (Post-stress). Total distance traveled during the 2hr test did not differ between UCMS (n = 9) and control (Con; n = 9) ship-with-dam rats before the start of UCMS. Adolescent ship-with-dam rats exposed to UCMS significantly increased ambulatory distance Post-stress. B) The increase in total distance traveled Post-stress in the

UCMS group was present after 15min of the Open Field test. C) Vertical time (time spent rearing) in the Open Field test increased over time, but was similar between groups. There was a trend for the UCMS group to rear more in the Post-stress Open Field test. D) The trend for increased vertical time Post-stress was not evident at 15min. E) Adolescent ship-with-dam rats exposed to UCMS spent more time in the center of the Open Field arena during the full 2hr test. F) Despite spending significantly more time in the center of the Open Field arena overall, UCMS rats distributed activities (percentage of time in the center) including resting (rest), rearing (vertical), engaging in fine movements (stereotypic), and ambulating (ambulatory) in a similar fashion as did controls. Values show group means ± SEMs. **$p \leq 0.01$, *$p \leq 0.05$, †$p \leq 0.08$ for between-group comparisons.

We examined anxiety-like behavior directly in the Elevated Plus Maze during week 5 of UCMS. The results are summarized in Table 3.1. In contrast to the increased exploratory behavior noted in the Open Field, UCMS-exposed adolescent rats did not differ from controls in any measures collected on the Elevated Plus Maze: number of open or closed arm entries, time spent in open or closed arms, or latency to first enter an open or closed arm [all $p>0.05$]. Thus, ship-with-dam adolescent rats exposed to UCMS during adolescence show no hyperactivity or reduction in anxiety-like behaviors compared to controls in the Elevated Plus Maze. These results are in striking contrast to reduced anxiety-like behaviors and increased activity on the Elevated Plus Maze that we found in wean-and-ship adolescent rats (**Chapter 2**).

Table 3.1. UCMS during adolescence does not affect behavior on the Elevated Plus Maze in ship-with-dam rats.

Anxiety-like behavior was assessed in the Elevated Plus Maze (EPM) during week 5 of UCMS. Adolescent ship-with-dam rats exposed to UCMS (n = 9) did not differ from controls (Con; n = 9) on any measures in the Elevated Plus Maze [all $p>0.05$], indicating no shift in anxiety-like behaviors in ship-with-dam rats. Means ± SEMs are shown for measures scored.

EPM Measure	Con	UCMS
Open Arm Entries	6.4 (0.8)	5.7 (0.8)
Closed Arm Entries	11.1 (1.5)	12.4 (0.8)
% Open Arm Entries	36.7 (4.6)	30.2 (3.5)
Time in Open Arms (s)	85.7 (15.1)	74.7 (10.2)
Time in Closed Arms (s)	150.1 (14.8)	164.7 (16.5)
% Time in Open Arms	28.6 (5.0)	24.9 (3.4)
Latency to Enter Open Arm (s)	49.4 (13.4)	35.9 (8.2)
Latency to Enter Closed Arm (s)	23.9 (10.9)	30.4 (8.0)

Together, our behavioral data indicate a profile of ship-with-dam adolescent rats exposed to UCMS during adolescence that is quite different from what we find when using wean-and-ship adolescent rats. Here, we see that UCMS exposure during adolescence has no impact on weight gain, does not cause anhedonia, and does not impact anxiety-like behavior in the Elevated Plus Maze. UCMS-exposed ship-with-dam adolescent rats did show signs of hyperactivity in the Open Field, but the effects were not as robust as what we observed in wean-and-ship rats (**Chapter 2**). We next aimed to examine whether the neuroendocrine changes we observed in wean-and-ship rats after UCMS exposure during adolescence were also dependent upon peri-weaning experience.

3.3.3 UCMS during adolescence does not cause neuroendocrine changes in ship-with-dam adolescent rats

To determine the effect of UCMS during adolescence on HPA axis function in ship-with-dam adolescent rats, we collected plasma upon termination of the study after the fifth week of UCMS in the same late-adolescent rats that underwent behavioral testing. Some rats (n = 3 UCMS, 3 Con) served as the BASELINE group; some rats (n = 3 UCMS, 3 Con) received saline injections 6hr prior to undergoing an acute forced swim stress and served as the acute stress group (sal/FST); and some rats (n = 3 UCMS, 3 Con) received an injection of the synthetic glucocorticoid dexamethasone 6hr prior to an acute FST and served as the negative feedback regulation group (DEX/FST). Acknowledging the small group sizes, we conducted non-parametric analyses. Table 3.2 summarizes corticosterone levels in all 3 conditions for UCMS-exposed and control ship-with-dam adolescent rats. UCMS and control ship-with-dam adolescent rats had similar baseline levels of circulating corticosterone [Wilcoxon Ram-Sum (W) = 11,

$p>0.05$]; circulating corticosterone levels were increased to a similar degree after an acute forced swim stress [$W = 12$, $p>0.05$]; and negative feedback did not appear to be impaired [$W = 12$, $p>0.05$]. Thus, UCMS exposure during adolescence in ship-with-dam adolescent rats does not cause the same neuroendocrine changes (lower absolute corticosterone levels at baseline and after an acute stress) that were observed in wean-and-ship adolescent rats exposed to UCMS (**Chapter 2**).

Table 3.2. UCMS during adolescence does not alter neuroendocrine levels in ship-with-dam rats.

HPA axis function was assessed after 5 weeks of UCMS (n = 9) or control conditions (Con; n = 9) in the same ship-with-dam adolescent rats from the behavioral experiments. There were no changes in basal (BASELINE; n = 3 UCMS, 3 Con), acutely-evoked (Sal/FST; n = 3 UCMS, 3 Con), or dexamethasone-suppressed (DEX/FST; n = 3 UCMS, 3 Con) corticosterone levels after UCMS exposure in adolescence [all $p>0.05$]. Corticosterone levels (ng/ml) are listed as group medians (range) for each condition.

Condition	Corticosterone (ng/ml)	
	Con	UCMS
BASELINE	79.0 (24.3-103.4)	28.0 (13.9-144.0)
Sal/FST	305.6 (241.9-332.2)	360.9 (135.6-407.5)
DEX/FST	32.0 (7.6-55.6)	14.6 (9.6-15.1)

3.3.4 Peri-weaning experience shifts behavioral baseline

Our findings using adolescent rats that experienced the stress of weaning and shipping separately (ship-with-dam) suggested that UCMS exposure during adolescence does not have any impactful consequences on adolescent rats, particularly with regard to anxiety-like behaviors. This is in stark contrast to findings with adolescent rats that experienced the stress of weaning and shipping concurrently (wean-and-ship), in which UCMS exposure during adolescence caused robust group differences in anxiety-like behavior on the Elevated Plus Maze (**Chapter 2**). A summary of the difference in findings between ship-with-dam and wean-and-ship adolescents is shown in Table 3.3. Both groups of adolescents were tested in parallel in our facility; the only difference between the two experiments was the peri-weaning experience of the adolescent rats. We therefore wanted to investigate whether different peri-weaning experiences could be responsible for the differential findings between the two groups. We conducted more detailed analyses of behavior, including early-life experience (ship-with-dam versus wean-and-ship condition) as an additional between-subjects factor, for the two measures with the most discrepancies in results: total weight gain and the Elevated Plus Maze. We also compared basal and acute stress-evoked corticosterone levels between conditions. Our results suggested that there was a more complex relationship than expected: in some cases the UCMS groups differed, suggesting a blunting effect of UCMS exposure during adolescence in ship-with-dam rats. In other cases, however, there were distinct differences between *control* groups.

Table 3.3. Peri-weaning experience influences the effects of UCMS exposure during adolescence.

Relative experimental outcomes of UCMS exposure during adolescence are compared for adolescent rats that had weaning and shipping stress separated in time (ship-with-dam rats; n = 9 UCMS, 9 Con) and adolescent rats that were weaned and shipped concurrently (wean-and-ship rats; n = 10 UCMS, 10 Con). Experiments were conducted in parallel, and the only difference between studies was the peri-weaning experience of adolescent rats. With the exception of Open Field hyperactivity, the effects of UCMS during adolescence were blunted in ship-with-dam rats compared to the effects observed in wean-and-ship rats. Abbreviations are as described previously. ↑↑significantly increased, ↓↓significantly decreased, compared to Con [$p<0.01$]; ↑significantly increased, ↓significantly decreased, compared to Con [$p<0.05$]; = no difference, for between-groups comparisons.

Measure	ship-with-dam Con vs UCMS	wean-and-ship Con vs UCMS
%Weight Gain	=	↓↓
SPT week 5	=	=
2hr OF Horizontal Exploration	↑↑	↑↑
2hr OF Vertical Exploration	=	↑
2hr OF Time in Center	↑↑	↑↑
EPM %Open Arm Time	=	↑
EPM Latency to 1st Open Arm	=	↓↓
BASELINE CORT	=	↓
Acute FST-evoked CORT	=	↓

Figure 3.5 shows total weight gain for ship-with-dam and wean-and-ship experiments together. When total weight gain for ship-with-dam and wean-and-ship conditions were analyzed together, there was a significant effect of UCMS [main effect of group: $F(1,34) = 9.88$], but no significant effect for peri-weaning experience [$F(1,34) = 0.88$, $p>0.05$] and no group x peri-weaning experience interaction [$F(1, 34) = 1.68$, $p>0.05$]. Examining the graph of total weight gain, it appeared that the ship-with-dam UCMS group had weight gain more similar to both control groups. This suggested that being shipped with a foster dam blunted the UCMS effect on weight gain. Because changes in weight can occur separately from other effects of UCMS (Flak et al., 2011), we examined the Elevated Plus Maze behavior across peri-weaning conditions next.

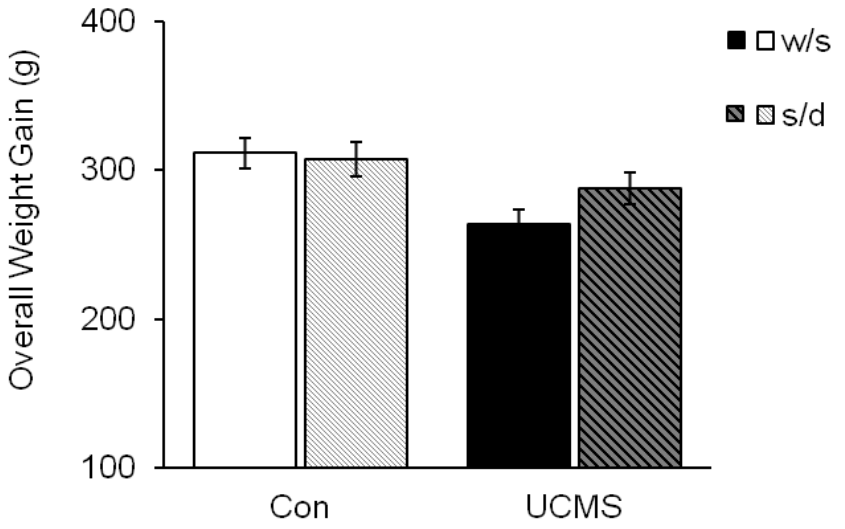


Figure 3.5. Peri-weaning experience impacts the effects of UCMS during adolescence on weight gain.

We conducted two-way ANOVAs with group (UCMS, Con) and peri-weaning experience (ship-with-dam, wean-and-ship) as between-subjects factors on total weight gain. The ship-with-dam UCMS rats (n = 9; dark grey stripes) did not differ from ship-with-dam controls (Con; n = 9; light grey stripes). As reported in **Chapter 2**, the wean-and-ship UCMS rats (n = 10; black bar) had significantly lower weight gain compared to wean-and-ship Con rats (n = 10; white bar). There was not a significant group x peri-weaning interaction [$p>0.05$] in this measure, but ship-with-dam UCMS weights tended to be more similar to control values. Values show group means ± SEMs.

Figure 3.6A shows percent open arm entries in the Elevated Plus Maze for ship-with-dam and wean-and-ship experiments together. We analyzed the percent of entries, to avoid confounds from the tendency of wean-and-ship UCMS rats to make more total entries. When percent open arm entries for ship-with-dam and wean-and-ship conditions were analyzed together, there was a significant group x peri-weaning experience interaction [$F(1, 34) = 6.86$, $p=0.013$]. UCMS rats made significantly more percent open arm entries, only in the wean-and-ship group [post-hoc $p=0.012$]. The ship-with-dam groups did not differ from each other [post-hoc $p=0.279$]. There was also a significant difference in percent open arm entries *between control groups*: ship-with-dam controls made a significantly higher percentage of open arm entries compared to wean-and-ship controls [post-hoc $p=0.021$]. UCMS-exposed ship-with-dam and wean-and-ship rats had similar percent open arm entries [post-hoc $p>0.05$]. We additionally found a significant group x peri-weaning experience interaction for percent time in the open arm [$F(1, 34) = 4.33$, $p=0.045$] (data not shown); post-hoc tests revealed that there was a significant difference in percent open arm time for wean-and-ship controls versus wean-and-ship UCMS rats only [$p=0.021$] (**Chapter 2**). Figure 3.6B shows latency to first enter the open arm. Because the latency data were not normally distributed, we transformed the data ($\log_{10}$) prior to statistical analyses. There was a significant main effect of group [$F(1,34) = 11.07$, $p=0.002$]. There was also a significant group x peri-weaning experience interaction [$F(1,34) = 4.99$, $p=0.032$]: UCMS reduced the latency to first enter the open arm compared to controls, only for the wean-and-ship adolescent rats [post-hoc t-test: $p<0.001$] (**Chapter 2**). Here, there was a difference between the two UCMS groups: the ship-with-dam rats had a significantly longer latency to first enter the open arm compared to the wean-and-ship rats [post-hoc t-test: $p=0.045$]. In all Elevated Plus Maze measures, the ship-with-dam controls tended to behave more similarly to UCMS-exposed rats than to wean-and-ship

controls. Together, these data provided behavioral evidence that suggested that being shipped with a foster dam was more stressful to an animal than being weaned and shipped at the same time, and also suggested inherent differences in activity between control groups.

Finally, we compared corticosterone levels at BASELINE and after an acute FST between ship-with-dam and wean-and-ship conditions, using nonparametric analyses. Notably, there was a significant group difference between ship-with-dam and wean-and-ship controls at BASELINE: ship-with-dam controls had significantly lower basal circulating corticosterone levels compared to wean-and-ship controls [$W = 18$, $p=0.029$]. There were no group differences in controls after an acute FST, nor were there any group differences between ship-with-dam and wean-and-ship UCMS groups [all $p>0.05$] (data not shown). Based on our previous findings that UCMS during adolescence results in lower circulating corticosterone levels (**Chapter 2**), this lends additional support to the notion that the ship-with-dam adolescents are already shifted to a more stressed state without UCMS, and indicates that UCMS is not additive in stress effects. Thus, environmental conditions around the time of weaning were sufficient to induce behaviors in the Elevated Plus Maze and neuroendocrine profiles that resembled those we observed in rats after 5 weeks of UCMS exposure throughout the course of adolescence.

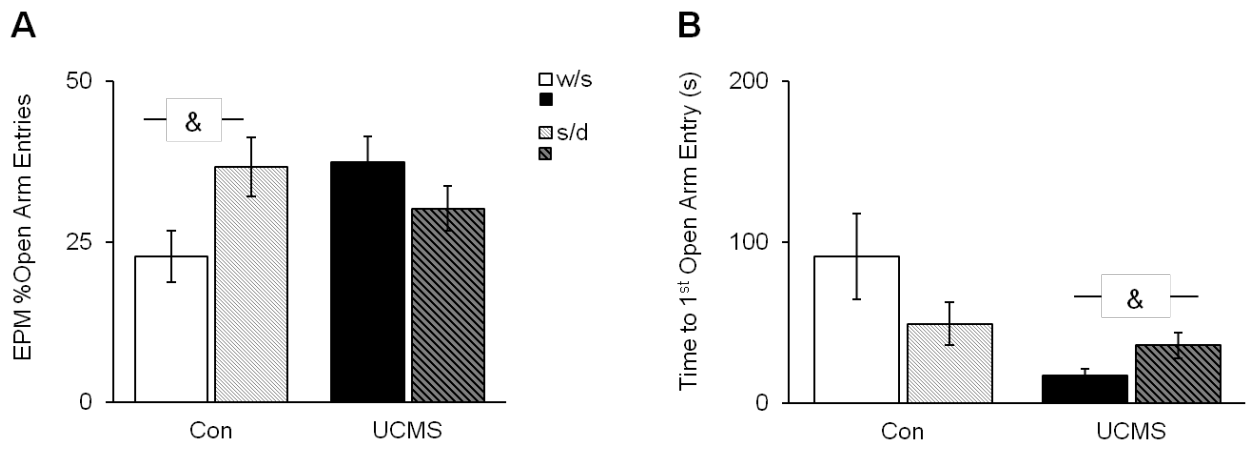


Figure 3.6. Peri-weaning experience affects anxiety-like behavior on the Elevated Plus Maze.

We conducted two-way ANOVAs with group (UCMS, Con) and peri-weaning experience (ship-with-dam, wean-and-ship) as between-subjects factors on behavioral measures in the Elevated Plus Maze. A) There was a significant group x peri-weaning experience interaction for percent open arm entries [calculated as (open arm entries / total arm entries) x 100]: adolescent ship-with-dam controls (n = 9; light grey stripes) had significantly more percent open arm entries compared to adolescent wean-and-ship controls (n = 10; white bars). The ship-with-dam control rats behaved similarly to both ship-with-dam UCMS rats (n = 9; dark grey stripes) and wean-and-ship UCMS rats (n = 10; black bars). B) There was also a significant group x peri-weaning experience interaction for latency to first enter the open arm: UCMS exposure reduced the latency to enter the open arm only in wean-and-ship condition. There was no difference between control and UCMS ship-with-dam rats [$p>0.05$]. Values show group means ± SEMs. &$p \leq 0.05$ for within-group comparisons.

Our analyses yielded evidence that suggested the adolescent rats that were shipped with a foster dam were more stressed than the adolescent rats that were weaned and shipped concurrently – often the ship-with-dam controls exhibited behavior that was more closely related to UCMS-exposed adolescent wean-and-ship rats than to control adolescent wean-and-ship rats. However, the effect was not additive: UCMS exposure did not increase activity levels any higher than what were observed in ship-with-dam controls. To investigate the possibility that peri-weaning experience altered rats' initial activity, prior to any additional UCMS exposure, we re-examined initial Open Field (pre-stress) activity for all rats. Prior to the start of any UCMS exposure, peri-weaning experience already impacted locomotor activity in a 2hr Open Field [independent samples t-test between peri-weaning experiences: $t(36) = 2.80$, $p=0.008$]. This difference in activity level depending on peri-weaning experience was evident within the first 15min of the pre-stress Open Field test as well [$t(36) = 5.16$, $p<0.001$]. Figure 3.7 shows total distance traveled in the Open Field for all ship-with-dam rats ($n = 18$) and wean-and-ship rats ($n = 20$) used in our experiments; again, at this point no rats had been exposed to UCMS. The ship-with-dam group also exhibited increased rearing times, compared to the wean-and-ship group, during the first 15min of the Open Field [$t(36) = 3.49$, $p=0.002$]; this pattern was maintained at a trend level at 2hr [$t(36) = 1.66$, $p=0.10$] (data not shown). Thus, prior to any UCMS exposure, the ship-with-dam rats were more hyperactive in the Open Field, and inherently resembled wean-and-ship rats that had experienced 5 weeks of UCMS.

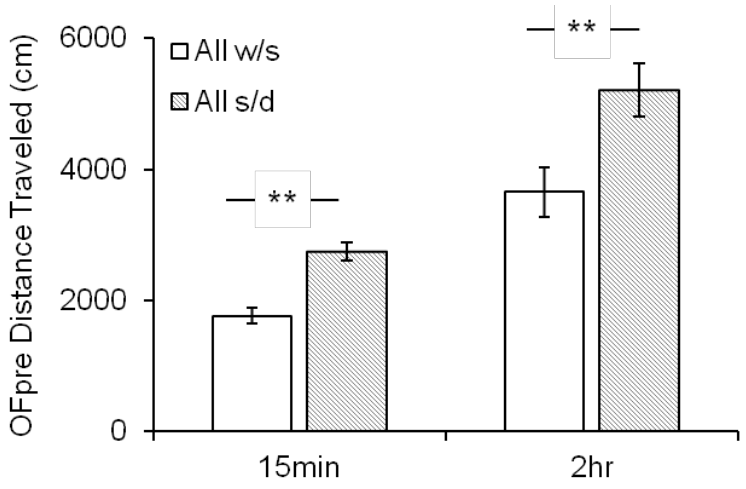


Figure 3.7. Peri-weaning experience shifts initial locomotor activity levels in the Open Field.

Prior to any UCMS exposure, rats that are shipped with a foster dam and weaned in-house (ship-with-dam; n = 18; grey bars) exhibit increased locomotor activity, i.e., distance traveled, in the Open Field at 15min and over the full 2hr test, compared to rats that are weaned and shipped concurrently (wean-and-ship; n = 20; white bars). This increased locomotor activity is similar to what is seen in wean-and-ship rats exposed to 5 weeks of UCMS and retested in the Open Field. Values show group means ± SEMs. **$p \leq 0.01$ for between-group comparisons.

3.3.5 Rats born in-house have activity levels similar to wean-and-ship rats

Because our more-detailed analyses suggested that the observed lack of a UCMS effect on adolescent ship-with-dam rats, in contrast to findings of robust UCMS effects on adolescent wean-and-ship rats (**Chapter 2**), may partially be due to a shift in phenotype of control rats, we conducted an additional experiment using rats that were born in our own facility (in-house controls). This in-house group experienced no shipping stress at all, and were run in parallel with additional ship-with-dam (n = 4) and wean-and-ship (n = 3) controls. Behavioral results for the additional ship-with-dam and wean-and-ship rats did not differ from the previously collected control data, so all control results were combined for final group sizes of (n = 9 in-house, 13 ship-with-dam, and 13 wean-and-ship controls). Rats were tested on the Open Field, as described above, shortly after weaning (see Figure 3.1). We will refer to this as pre-stress Open Field test for consistency with above results. Figure 3.8 shows total distance traveled in an Open Field in the three control groups. Again, we found evidence for the ship-with-dam condition to exhibit locomotor hyperactivity in the Open Field. There was a main effect of peri-weaning experience [Two-way ANOVA: $F(2,32) = 6.83$, $p=0.003$]. The ship-with-dam adolescents traveled significantly more than wean-and-ship adolescents [post-hoc t-test: $p=0.018$], and significantly more than the in-house adolescents [post-hoc t-test: $p=0.007$]. Total distance traveled was comparable between the wean-and-ship and in-house adolescents [post-hoc t-tests, both $p>0.05$]. The same pattern of differences was evident in the first 15min of the Open Field: there was a main effect of peri-weaning experience [$F(2,32) = 11.3$, $p<0.001$], and post-hoc comparisons showed that ship-with-dam adolescents traveled more than the wean-and-ship adolescents [$p<0.001$] and more than the in-house adolescents [$p=0.012$]. The locomotor activity of the wean-and-ship adolescents never significantly differed from that of the in-house adolescents

[both $p>0.05$]. Thus, there was something inherently different about being shipped with a foster dam that increased locomotor activity, compared to rats weaned and shipped, or rats born in-house with no shipping stress at all. These results suggest that being shipped with a foster dam, to avoid compounding the stressors of weaning and shipping, may actually be more stressful to adolescent male rats.

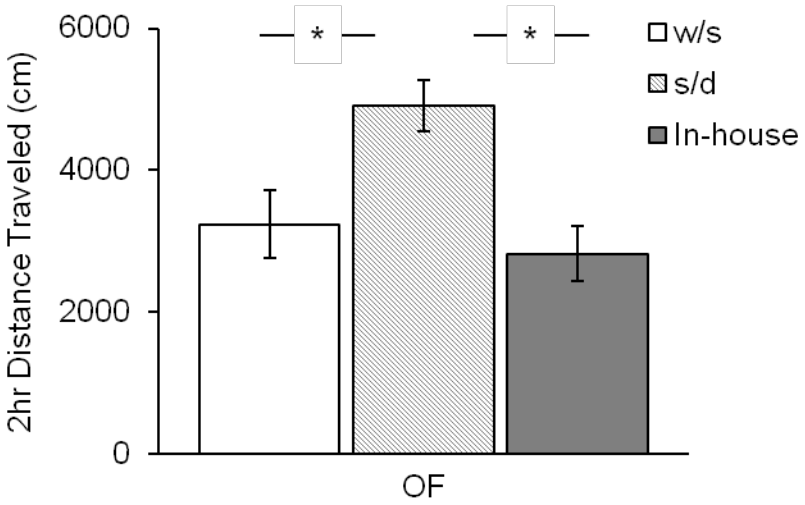


Figure 3.8. Adolescent rats born in-house have similar distance traveled in the Open Field as adolescents weaned and shipped concurrently.

We compared behavior in an Open Field test between control animals that were weaned and shipped concurrently (wean-and-ship; n = 13; white bar), shipped with a foster dam (ship-with-dam; n = 13; light grey bar), or born in-house (n = 9; dark grey bar). A) Adolescent rats that were shipped with a foster dam demonstrated similar locomotor behavior in the Open Field as what had previously been observed in wean-and-ship adolescent rats exposed to 5 weeks of UCMS during adolescence: total distance traveled over 2hr was significantly higher than either wean-and-ship controls or in-house controls. Values show group means ± SEMs*$p<0.05$ for between-group comparisons.

3.4 DISCUSSION

We previously demonstrated robust hyperactivity and decreased anxiety as a consequence of unpredictable chronic mild stress (UCMS) exposure during adolescence in adolescent rats (**Chapter 2**). Here, we attempted to replicate our findings using adolescent rats that had a different peri-weaning experience. The current study differed from our previous study in the following ways. Previously, adolescent rats were weaned and shipped concurrently on postnatal day 21 (p21). Here, adolescent rats were shipped in cohorts of 8 male pups with 1 foster dam on p18. Upon arrival, rats and dams were left undisturbed until weaning occurred in-house on p21. Thus, while both peri-weaning conditions had a weaning date of p21, the rats shipped with a dam had the stress of shipping separated in time from the stress of weaning. Rats raised at the supplier were exposed to group-housing conditions with multiple dams assigned to groups of same-age, same-sex pups, so we believed the ship-with-dam condition would be less stressful than the wean-and-ship condition we used previously. With this different peri-weaning experience, we obtained substantially different results. UCMS exposure during adolescence in pups that were shipped with a foster dam on p18 and weaned in-house on p21resulted in no changes in weight gain, no shift in anxiety-like behaviors, and no change in basal or acute stressor-evoked corticosterone levels relative to control adolescents. Viewed alone, the current results suggest that an earlier shipment age with a dam protects against the negative consequences of UCMS during adolescence. However, when results of the current study (earlier shipment with dam) were compared more closely to our findings reported in **Chapter 2** (weaned and shipped concurrently), this parsimonious interpretation may prove to be incorrect.

At first glance, when comparing the UCMS effects between peri-weaning conditions, our results imply that the concurrent wean-and-ship condition is more detrimental to adolescents,

whereas allowing young rats to experience the stress of shipping separately from that of being weaned might be somewhat protective. We observed clear behavioral and neuroendocrine effects of UCMS exposure during adolescence in the wean-and-ship condition, compared to controls, and with the exception of hyperactivity in the Open Field, the UCMS effects were lost in the ship-with-dam condition. Studies on foster experiences in humans suggest there could be a protective effect of foster care on stress axis function, in that foster care was shown to reverse the blunted cortisol levels observed in children with a prior history of abuse (Gunnar & Quevedo, 2008). Similarly, research in rodents indicates that cross-fostering may reverse detrimental effects of prior maternal stress in young rats, and may prevent depressive-like behaviors in adulthood [e.g.: (Friedman et al., 2006; Maccari et al., 1995)]. Weaning and shipping the rats concurrently could be causing something akin to a "two-hit" model to occur: the initial small bit of stress (weaning and shipping) could prime the young adolescents to be more susceptible to future stress insults (e.g., the UCMS exposure during adolescence). However, if this were the case, then it would be more likely that the wean-and-ship UCMS group would demonstrate *worse* experimental outcomes (additive effect from the early life stressor) than would the ship-with-dam UCMS group; and this was not the case.

However, upon closer inspection of the peri-weaning experiences, there lies a more complex relationship between the two conditions. Comparing the results between the ship-with-dam and wean-and-ship UCMS groups, it seems that there may not have been a "protective" effect of the ship-with-dam condition: most of the behavioral data in the two UCMS-exposed conditions did not differ statistically from one another, i.e., the ship-with-dam condition did not significantly *prevent* any of the UCMS-induced behavioral changes. Additionally, in some cases, the ship-with-dam *control*s exhibited behavioral profiles that tended to be more similar to the

UCMS groups than to either the wean-and-ship control group or, in the case of the Open Field test, the adolescents that were born in-house (Figure 3.8). This goes against the idea that allowing young rodents to experience the stress of shipping together with a dam and other pups *decreases* the shipping-associated stress in those young rodents.

Our results led us to conclude that male early-adolescent rats weaned and shipped concurrently may be a reasonable alternative when in-house breeding facilities are not an option. However, we did not investigate whether the consequences of UCMS on adolescents that were born in-house are more similar to those observed in adolescents weaned and shipped on p21 or to those observed in adolescents shipped on p18 and weaned in-house on p21. Although the outcome for the latter two groups did not differ statistically, there was a trend for the UCMS effects to be blunted in the group shipped with a foster dam compared to the group weaned and shipped on the same day. If findings showed that the UCMS effect is yet more blunted in in-house born adolescents than adolescents shipped with a foster dam, then the concern that weaning and shipping on the same day sets up a "two-hit" situation would be more justified. Clearly, more work is needed to determine whether peri-weaning experiences could serve to be protective or detrimental later in life.

There are many well-studied paradigms of early-life stress that are much more clear in the characterization of the behavioral, neuroendocrine, and neurobiological consequences of stress around the age of weaning. For example, in rodents, prolonged periods of deprivation of maternal care (1-3hr) have been shown to cause depressive- and anxiety- like behaviors and over-active HPA axis function in adolescence and adulthood (Anisman et al., 1998; Francis et al., 1999; Ladd et al., 2004; Workel et al., 2001). Also, environmental perturbations in pre-weanling rats have been shown to reduce glucocorticoid receptor gene expression in the

paraventricular nucleus of the hypothalamus, while also increasing basal corticosterone levels, in adulthood (Avishai-Eliner et al., 2001; Gilles et al., 1996).

3.4.1 Conclusions

In conclusion it is clear that experiences surrounding the age of weaning can cause alterations at the behavioral, neuroendocrine, and neurobiological levels. However, more well-controlled parametric studies, in which mutiple outcome measures covering neural, endocrine, and behavioral domains are included, are needed for the field to agree on acceptable rearing practices for adolescent work.

4.0 LOCOMOTOR RESPONSE TO NOVELTY IS NOT A STABLE ATTRIBUTE IN ADOLESCENCE

4.1 INTRODUCTION

Differences in the way individuals respond to life experiences are being recognized as important variables to be studied. Variations in response strategies, particularly with regard to response to stress, are being examined in the hopes of identifying those individuals most at risk to develop neuropsychiatric disorders, including depression, anxiety, and drug addiction [e.g.: (Ersche et al., 2012; Tarter et al., 2012)]. Researchers have begun to utilize animal models in order to understand the neurobiology of those identified traits (or attributes) associated with increased vulnerability, or resilience, to disease (Belin et al., 2011; Flagel et al., 2010; Piazza et al., 1989; Stead et al., 2006).

One such behavioral attribute that has implications in both mood disorders and addiction is novelty-seeking (Bardo et al., 1996; Calvo et al., 2011; Dawe et al., 2004; Klebaur et al., 2001; Stedenfeld et al., 2011). Novelty-seeking behavior is defined as the increased exploration of novel situations or stimuli (Vidal-Infer et al., 2012). Individual differences in novelty-seeking behavior in adults, typically assessed in terms of locomotor activity in a novel environment, have been found to correlate with differences in the tendency to develop stress-induced trait anxiety, and depressive-like, or addictive behaviors. Specifically, adult rodents that display very low

levels of locomotor activity in a novel environment (Low Responders) are prone to develop anxiety- and depressive-like behavior after stress, whereas those that exhibit very high levels of locomotor activity (High Responders) are prone to exhibit reduced inhibitory control, elevated risk-taking, and addictive behavior (Belin et al., 2011; Calvo et al., 2011; Dellu et al., 1996; Piazza et al., 1991). In fact, investigators have now created a genetic line of the High- and Low-Responders, by breeding those rats with the highest locomotor activity together, and those with the lowest locomotor activity together over many generations, denoted bred-High/bred-Low-Responders (Stead et al., 2006). These genetic lines have repeatedly been demonstrated to have differential vulnerabilities: bred-High Responders are more prone to exhibit addictive-like behaviors, with accompanying changes in striatal dopamine levels; and bred-Low Responders are susceptible to Unpredictable Chronic Mild Stress-induced depressive-like behaviors and neuroendocrine profiles [(Davis et al., 2008; Flagel et al., 2010; 2008; 2006; Stedenfeld et al., 2011); reviewed in: (Flagel et al., 2014)].

Adolescence is characterized as a time of increased exploration and risk-seeking, and adolescent rats have been shown to be more responsive to novel stimuli compared to adults (Douglas et al., 2003, 2004; Spear, 2000). Adolescence in rodents can be divided into stages anchored around pubertal development, corresponding to early- (postnatal day [p] 21-34), mid- (p35-45), and late-adolescence (p46-60), (Eiland & Romeo, 2013; McCormick & Mathews, 2007; McCormick et al., 2010; Spear, 2000; Tirelli et al., 2003). Adolescence is inherently a period of much change, as an individual transitions from youth into adulthood (Spear, 2000). Much neurodevelopment is occurring during adolescence, including substantial remodeling of brain regions implicated in mood/disorders and anxiety (e.g., the prefrontal cortex, the

hippocampus, the amygdala, and the nucleus accumbens), as well as the dopaminergic system (Casey et al., 2010; Lupien et al., 2009; Malter Cohen et al., 2013; Rubinow & Juraska, 2009).

Although many investigators have confirmed the vulnerable state of brain systems during adolescence in rodents (McCormick & Mathews, 2007, 2010; McCormick et al., 2010; Spear, 2000), few studies have assessed whether differential novelty-seeking behaviors in adolescent animals translate to differing degrees of susceptibility to stress-induced general anxiety and depression at one end of the continuum, and risk-taking and addiction at the other. In the few studies in which locomotor response to novelty was examined in young rats, the investigators failed to determine whether initial locomotor rankings are stable across time [e.g.: (Clinton et al., 2008; Oztan et al., 2011b; 2011a)]. It is critical to examine whether rankings of locomotor activity in a novel environment remain relatively constant across an animal's lifetime, to confirm that it is in fact a stable attribute, or "trait," suitable for use as a predictive variable. Here, we aimed to understand whether locomotor response to a novel environment (2hr Open Field test) was a stable attribute in adolescent and adult rats.

4.2 MATERIALS AND METHODS

4.2.1 Animals

In this study, we examined the test-retest reliability of a 2hr Open Field test (described below) in early adolescent and adult rats. We defined rodent adolescence as a broad age range that started a few days post-weaning (postnatal day 24; p24), spanned through puberty, and ended around p60. This range included Spear's (2000) conservative definition anchored around puberty and

behavioral changes (p28-42), as well as the periods that are considered to be early and late adolescence (McCormick & Mathews, 2007; Tirelli et al., 2003). All rats were housed in pairs in plastic cages (40 x 22 x 19cm) upon arrival, with standard rat chow (Purina) and water available *ad libitum*. Rooms were maintained on a standard 12hr light-dark cycle (lights on at 0700) in a temperature and humidity controlled environment. The University of Pittsburgh Institutional Animal Care and Use Committee approved all procedures used.

4.2.1.1 Adolescents

Twenty-four male Sprague-Dawley rats (Charles River, Portage, MI) were weaned and shipped from the supplier on p21. Upon arrival, rats were immediately housed in pairs, and left undisturbed until behavioral testing began in early adolescence on p23/24. These rats were part of a larger study that involved weekly sucrose preference tests, but were otherwise left undisturbed except for daily weighing and weekly cage changes. Note that all adolescents were exposed to CONTROL conditions.

4.2.1.2 Adults

Twenty-four male Sprague-Dawley rats (Charles River) arrived from the supplier on p60. Upon arrival, rats were immediately housed in pairs, and left undisturbed until behavioral testing began in adulthood on p73-75. These rats were part of a larger study that involved weekly sucrose preference tests, but were otherwise left undisturbed except for daily weighing and weekly cage changes. Note that all adults were exposed to CONTROL conditions.

4.2.2 Behavioral Procedures

Figure 4.1 shows the timeline for behavioral testing. Rats underwent only one behavioral assessment per day. All tests occurred in a single behavioral testing room during the light cycle. Rats were transported in their home cages and were allowed 5-10min to habituate to the testing room before starting. Initial tests for adolescent rats were conducted shortly after weaning (within one week of arrival; early adolescence). Adolescent rats then were left generally undisturbed in their home cages for ~5weeks until the second round of behavioral tests in late adolescence. Initial tests for adult rats were conducted ~2weeks after arrival. Adult rats then were left generally undisturbed in their home cages for ~8weeks until the second round of behavioral tests.

Figure 4.1. Experimental details.

Experimental Timeline. Age in postnatal days (p) for each test is indicated. Adult (n = 24) and adolescent (n = 24) rats were tested in the Open Field soon after arrival to the facility. Adults were given a second test within a week; a subset of adolescents (n = 8) was re-tested 1 day later. All rats were then left undisturbed in their home cages except for daily weighing, and weekly cage changes and sucrose preference tests (as part of another study). Rats were tested a final time in the open field much later (~5 weeks for adolescents; ~8 weeks for adults). OF: Open Field Test.

4.2.2.1 Open Field Test

The Open Field is a measure of exploratory and anxiety-like behavior in rodents (Brooks & Dunnett, 2009; Choleris et al., 2001; Coelho et al., 2014; Schmitt & Hiemke, 1998), but can also be used in a longer protocol (1-2hr) to assess individual differences in stress response to a novel environment (Calvo et al., 2011; Jama et al., 2008; Kabbaj et al., 2000; Stead et al., 2006; Stedenfeld et al., 2011). Rats were placed into an open chamber (43 x 43cm; MedAssociates, St. Albans, VT) under dim lighting (<10lux). Activity in the horizontal and vertical planes was monitored for two hours, via horizontal and vertical infared beam breaks across 16 evenly-spaced squares. Adolescent rats were tested in early adolescence on p23/24 (OF1); a subset (n = 8) were re-tested on p24/25 (OF2). All adult rats were given two tests on p73-75 (OF1) and p79-81 (OF2). Rats were tested a final time (OF3) approximately 5 weeks (adolescents; p58/59) or 8 weeks (adults; p130-132) later. Because novelty of the environment is inherent in the Open Field test and rats were tested multiple times, visual, tactile, and olfactory cues were changed between each Open Field exposure. The total distance traveled during the two hours provided an index of overall locomotor activity.

4.2.3 Statistical Analyses

Locomotor activity (i.e., distance traveled in cm) was recorded each minute across the full 2hr Open Field test. Individual rats' total distance traveled (sum across 2hr) was ranked from highest to lowest, and divided into tertiles to identify High Responders (top 1/3 of distribution) and Low Responders (bottom 1/3 of distribution) in adults and adolescents separately. Correlations of total distance traveled were calculated in adolescents and adults separately. Correlations were determined for tests conducted close in time (OF1 vs. OF2; 1-6 days in between), and for tests

conducted far apart in time (OF1 vs. OF3; 5-8 weeks in between). Additional t-tests with group (adolescent, adult) were conducted to compare time to habituate (defined as the time until distance traveled was ≤1/2 the total distance traveled during the first 5min of the test). Additional two-way analyses of variance (ANOVAS) with responder (adolescent, adult High Responders, adult Low Responders) were also conducted. Statistical analyses were performed using the SPSS software package version 20.0 (SPSS, Chicago, IL). Significance level was set to $\alpha \leq 0.05$.

4.3 RESULTS

4.3.1 Locomotor activity in a novel environment is a stable attribute in adult rats

To determine whether locomotor response to a novel environment (Open Field chamber) is a stable behavioral attribute, we tested adolescent and adult rats in an Open Field test up to three times: all were tested shortly after arrival (OF1), some were re-tested 1-6 days later (OF2), and all were re-tested 5-8 weeks later (OF3). Because only a subset of adolescent rats was tested in OF2 (n = 8), our major analyses focus on OF1 and OF3 (corresponding to early adolescence and late adolescence). Total distance traveled in the initial Open Field test (OF1) was ranked from highest to lowest in adults and adolescents separately, and divided into tertiles. High Responder and Low Responder groups were created from the top 1/3 and bottom 1/3 of the distribution, respectively. We first examined whether total locomotor activity (i.e., total distance traveled) correlated from one test to the next. The High/Low-Responder distinctions based on OF1 activity remained constant for adults – relative levels of distance traveled in OF1 for individual rats were similar to relative levels of distance traveled in OF2 and OF3. Figure 4.2 shows correlations for

Open Field distance traveled in adults for tests conducted 6 days apart (Figure 4.2A) and for tests conducted ~8weeks apart (Figure 4.2B), with individual data points for High Responders colored in black, and Low Responders colored in grey. The total distance traveled in OF1 was significantly correlated with total distance traveled in OF2 [$r = 0.893$, $p<0.001$] and with OF3 [$r = 0.751$, $p<0.001$]. These results suggest that locomotor response to a novel environment over the course of a 2hr test is a stable attribute in adult rats.

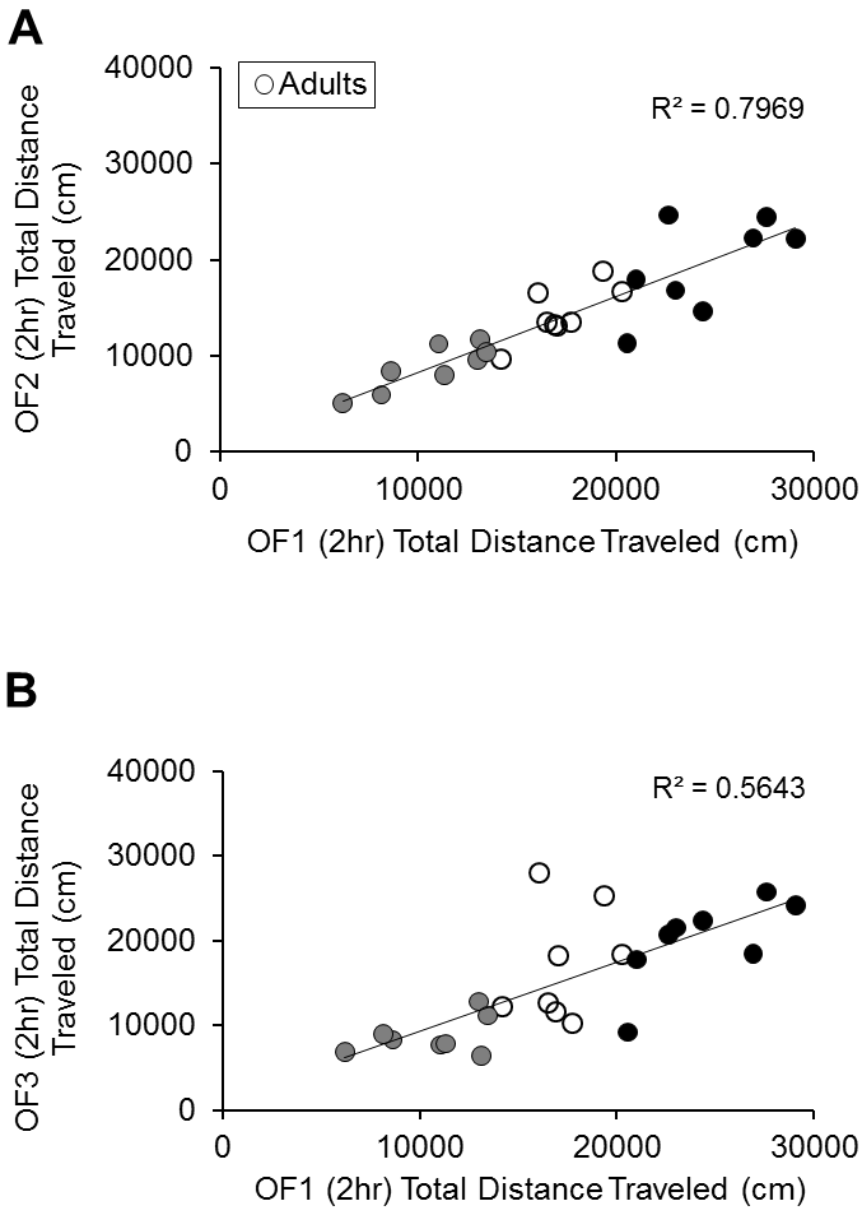


Figure 4.2. Adult locomotor activity in the Open Field is highly correlated over time.

Total distance traveled in a novel environment (OF1) of adult rats (n = 24; circles) was highly correlated with: A) total distance traveled in a second exposure to a novel environment (OF2), 6 days after OF1 [r = 0.893, p<0.001]; and B) total distance traveled in a third exposure to a novel environment (OF3), 8 weeks after OF1 [r = 0.751, p<0.001]. Graphs show individual data points and regression lines for all 24 rats. High Responders (determined in OF1 as top 1/3 of distance traveled) are colored in black, and Low Responders (determined in OF1 as bottom 1/3 of distance traveled) are colored in grey, for ease of comparison.

4.3.2 Locomotor activity in a novel environment is not a stable attribute in adolescent rats

In contrast to the robust reliability of relative total distance traveled levels in adult rats, adolescent rats had variable relative levels of total distance traveled across multiple Open Field tests. Figure 4.4A shows correlations for Open Field distance traveled in a subset of early adolescents for tests conducted one day apart (only 8 of the 24 adolescents were tested in OF2). Adolescent levels of distance traveled were not correlated [$r = 0.464$, $p>0.05$]. Additionally, when all adolescents were re-tested in the Open Field test 5 weeks after the initial Open Field test (OF3; late adolescence), the distance traveled was not correlated whatsoever [$r = -0.153$, $p>0.05$] (Figure 4.4B). Those rats designated as Low Responders in the initial Open Field test (OF1) traveled a much greater distance in OF3 than many of the rats initially designated as High Responders traveled in OF3. Thus, these results indicate that locomotor activity (total distance traveled) to a novel environment is not a stable behavioral attribute in adolescent rats and, hence, should not be considered as an organizing variable for young animals.

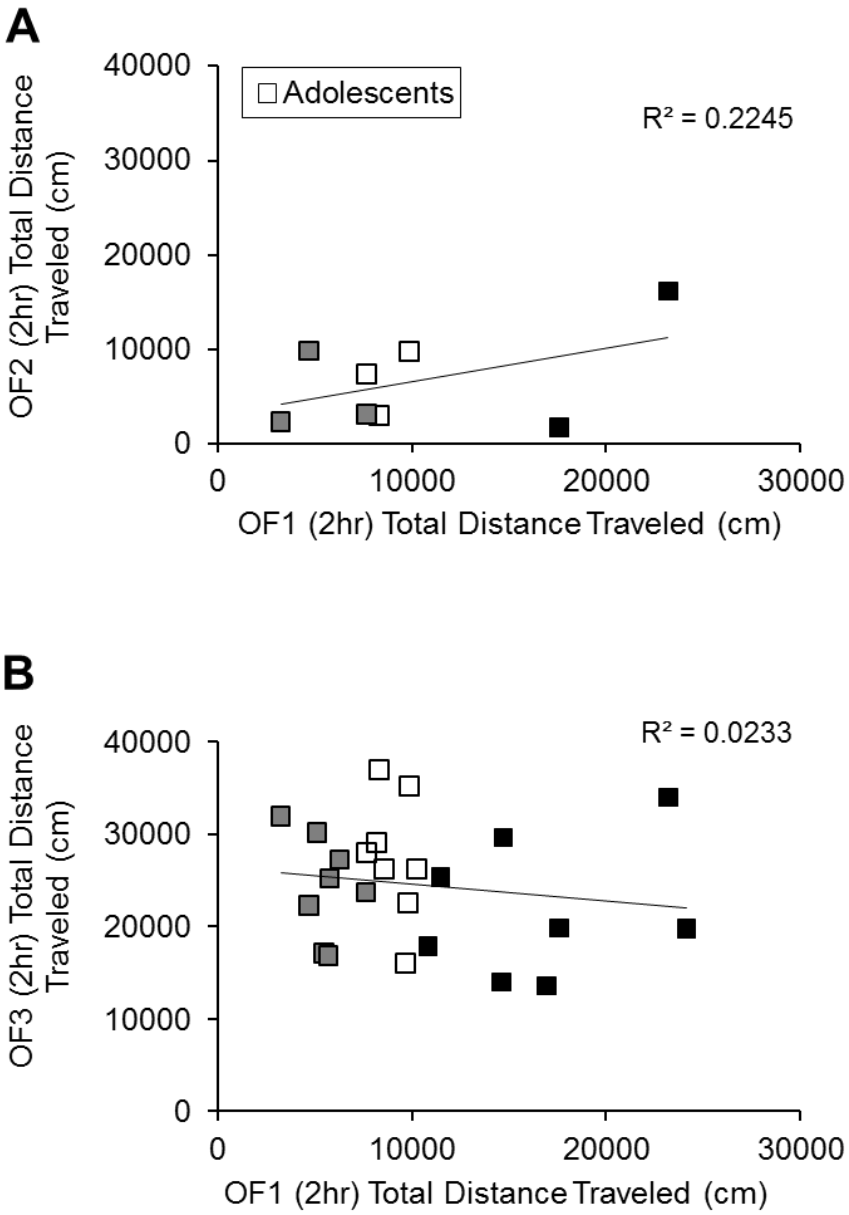


Figure 4.3. Adolescent locomotor activity in the Open Field is not correlated over time.

Total distance traveled in a novel environment (OF1) of adolescent rats (n = 24; squares) was not correlated with: A) total distance traveled in a second exposure to a novel environment (OF2), 1 day after OF1 [(n = 8), r = 0.464, p>0.05]; or B) total distance traveled in a third exposure to a novel environment (OF3), 5 weeks after OF1 [(n = 24), r = -0.153, p>0.05]. Graphs show individual data points and regression lines for all rats exposed to each test. High Responders (determined in OF1 as top 1/3 of distance traveled) are colored in black, and Low Responders (determined in OF1 as bottom 1/3 of distance traveled) are colored in grey, for ease of comparison.

4.3.3 Time to habituate locomotor response to a novel environment differs between early adolescents and adults

After identifying such inconsistencies in adolescent distance traveled in response to a novel environment, we wanted to examine the locomotor profile during the Open Field more closely, to determine whether adolescents and adults differed in other respects as well. Notably, other groups have indicated a separation between behaviors during the early portion of an Open Field test (first 5-10min; habituating to the novelty) and behaviors for the remaining portion of the Open Field test (from 10min on to the end of the 1-2hr test) (Arenas et al., 2014; Mathews et al., 2010; Philpot & Wecker, 2008). Thus, we chose to examine whether there were differences between adolescents and adults in their activity during the first 5min, and we also examined where there were differences in the rate of decline in locomotor activity, i.e., habituation, to the Open Field environment.

As shown in Figure 4.4A, distance traveled was greatest during the first 5min of the OF1 test. Activity declined rapidly during the first 15-30min, and reached asymptotic levels towards the second half of the test. Adolescents traveled significantly less than adults in OF1, during the first 5min [$t(46) = 5.81$, $p<0.001$] (Figure 4.4B), and also over the full 2hr Open Field test [$t(46) = 4.1$, $p<0.001$] (data not shown, but see Figure 4.6A). To rule out the possibility that the decreased movement in the adolescents was due to fatigue, we compared asymptotic levels of locomotor activity. We defined asymptotic level to begin at the first time bin (5min bins) of 3 consecutive bins that did not statistically differ from the distance traveled in the last 5min of the test; we then averaged the distance traveled (from point of asymptote to end of 2hr test) to determine asymptotic level of locomotor activity. Importantly, as Figure 4.4C demonstrates, adolescents and adults did not significantly differ in their level of asymptotic locomotor activity

[t(46) = 0.18, p>0.05]. This argues against the possibility that the younger animals reduced locomotor activity because of fatigue. Finally, we defined "habituation" as the time bin (5min bins) in which total distance traveled was less than or equal to 1/2 the distance traveled in the first 5min of the Open Field test (1/2Max). We thus calculated habituation time in three steps: 1) we determined the maximum distance traveled [sum of distance traveled from 0-5min]; 2) we determined 1/2Max [(sum 0-5min)/2]; and 3) examined the sum of distance traveled across 5min time bins until we identified the first bin that was equal to or below the 1/2Max value. We chose to sum the data over 5min bins to avoid large variability from minute-to-minute fluctuations in activity. Figure 4.5A shows the time to reach 1/2Max, i.e., time to habituate, for adolescents and adults in OF1. Adults required significantly more time to reach the habituation criterion compared to adolescents [t(46) = 4.14, p<0.001] (Figure 4.5A). This suggests that the adolescents' locomotor activity habituated more quickly to the novel environment than did the adults' locomotor activity. Interestingly, adult and late-adolescent rats had similar times to reach habituation in OF3 [t(46) = 0.11, p>0.05] (Figure 4.5B). This suggests that, whatever the underlying neurobiological processes involved in locomotor response to a novel environment might be, they may be developing to maturity from early adolescence through late adolescence.

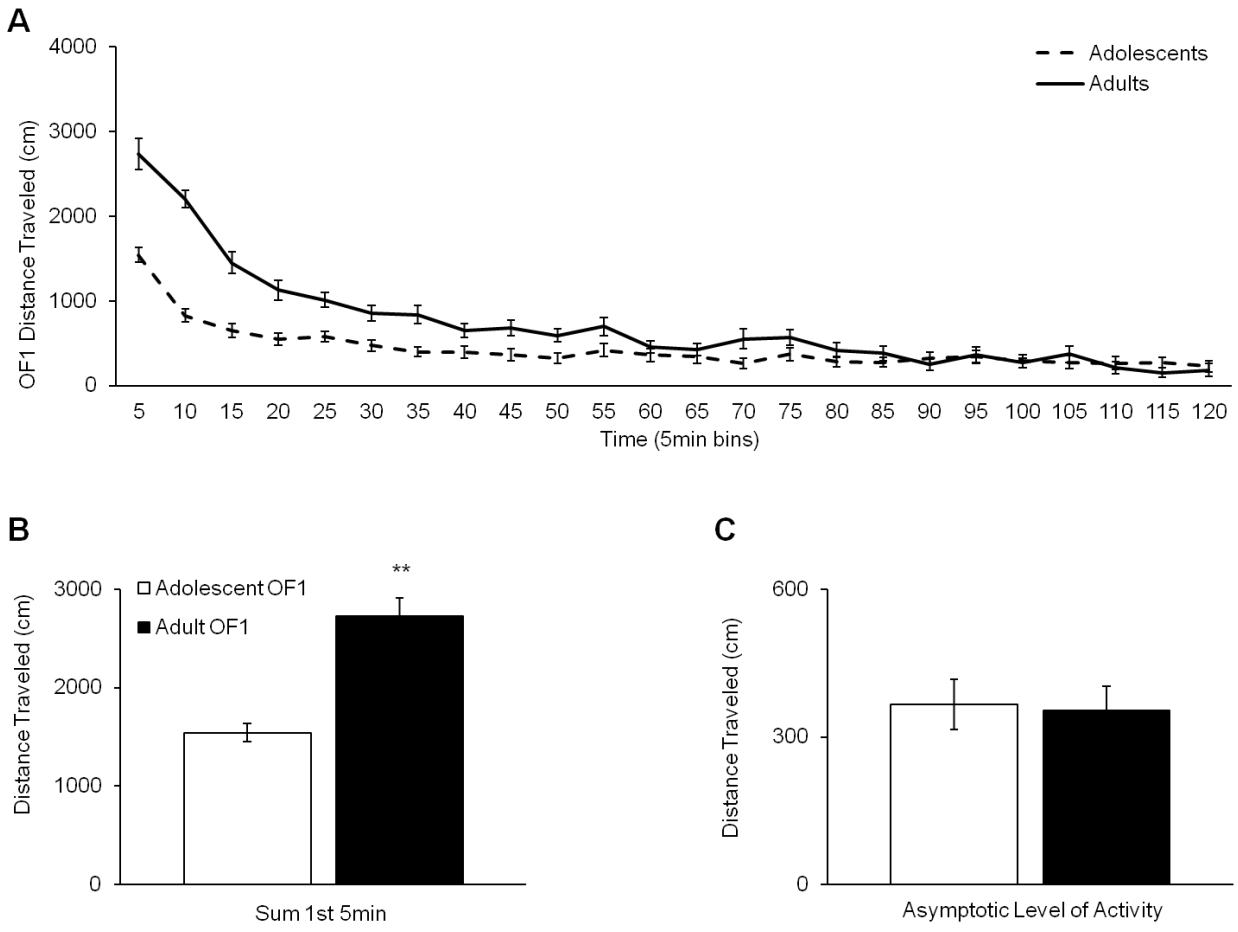


Figure 4.4. Initial locomotor activity in OF1 is higher in adults, but falls to similar levels as adolescents over time.

A) Adolescent rats (n = 24) and adult rats (n = 24) display similar patterns overall in locomotor activity during a two-hour period in a novel Open Field environment (OF1). Activity tapers down over the course of the 2hr test. B) Absolute levels of distance traveled (cm) were significantly lower in adolescents compared to adults in the first 5min of the Open Field test. C) Asymptotic levels of locomotor activity (see text for operational definition) did not significantly differ between age groups [$p>0.05$], indicating that adolescent/adult differences were not due to fatigue of the adolescents. Values show group means ± SEMs. **$p<0.01$ for between-group comparisons.

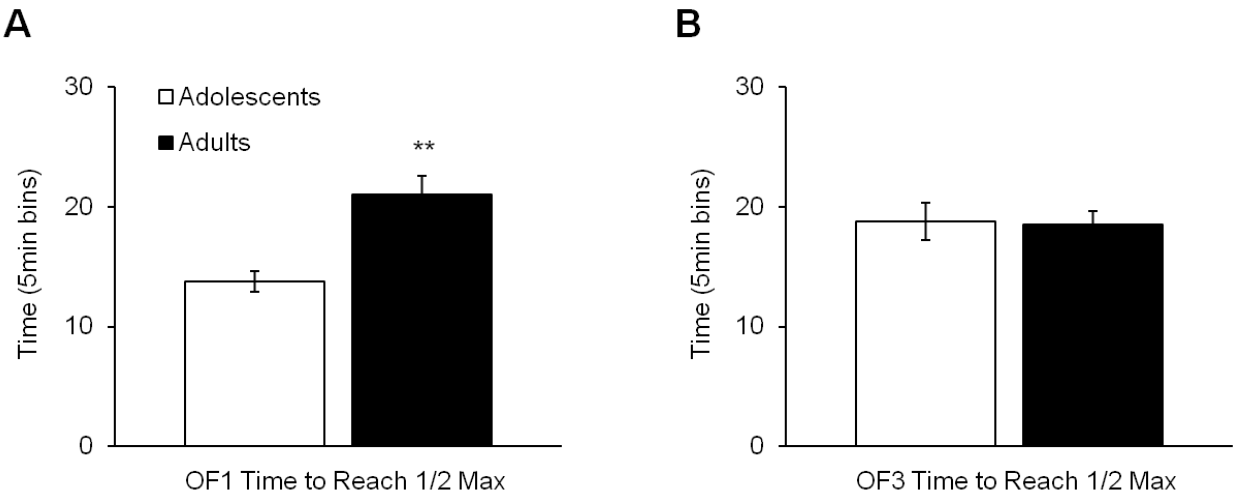


Figure 4.5. Locomotor activity in the OF1 habituates faster in adolescents than in adults.

A) Habituation of locomotor activity (see text for operational definition) differed between early adolescents (n = 24) and adults (n = 24) in an initial Open Field test (OF1). Adults took significantly longer to reach habituation criterion than adolescents. B) Habituation did not significantly differ between late adolescents and adults re-tested in a subsequent Open Field (OF3) [$p>0.05$]. Values show group means ± SEMs. **$p<0.01$ for between-group comparisons.

4.3.4 Early-adolescent locomotor activity resembles that of adult Low Responders, and late-adolescent locomotor activity resembles that of adult High Responders, in the Open Field

Upon separating adults into tertiles based on levels of total distance traveled in the initial Open Field (OF1), it was clear that the early adolescents' locomotor activity profile was similar to that of the adult Low Responders (Figure 4.6A). Distance traveled in the early adolescent rats over the course of the 2hr test in OF1 resembled the activity pattern of the adult Low Responders group (Appendix Figure A.2). Additionally, when we examined the late adolescent activity in the subsequent Open Field (OF3), the distance traveled over the course of the 2hr test resembled the activity pattern of the adult High Responders group (Figure 4.6B and Appendix Figure A.3). We conducted a two-way ANOVA on total distance traveled, with group (adolescent, adult High Responders, adult Low Responders) and test (OF1, OF3) as the between-subjects factors. There was a main effect of group [$F(2,74) = 23.11$, $p<0.001$], and a trend for a main effect of test [$F(1,74) = 3.81$, $p=0.055$], where distance traveled in OF3 was slightly higher than in OF1 (as we discuss below, this difference was due to the drastic increase in activity by the adolescent group). More importantly, there was a significant group x test interaction [$F(2,74) = 25.66$, $p<0.001$]. Post-hoc comparisons revealed that, in OF1, early-adolescent distance traveled did not significantly differ from adult Low Responders [$p>0.05$], but was significantly less than adult High Responders [$p<0.001$]. In OF3, late-adolescent distance traveled was significantly higher than adult Low Responders [$p<0.001$], but did not significantly differ from adult High Responders [$p>0.05$]. Additionally, when examining the overall shift in distance traveled from OF1 to OF3, neither adult Low Responders nor High Responders significantly changed their

total distance traveled from OF1 to OF3 [both post-hoc $p>0.05$]. However, adolescents significantly increased total distance traveled from OF1 to OF3 [$p<0.001$].

Interestingly, there were also adolescent similarities with adult Low Responders in OF1, and adult High Responders in OF3, when we examined the time to reach habituation (as defined above) (Table 4.1). We conducted a two-way ANOVA on time to reach habituation, with group (adolescent, adult High Responders, adult Low Responders) and test (OF1, OF3) as the between-subjects factors. There was a main effect of group [$F(2,74) = 4.50$, $p=0.014$], and there was a significant group x test interaction [$F(2,74) = 3.10$, $p=0.05$]. Post-hoc comparisons revealed that early adolescents took a similar amount of time to reach the habituation criterion in OF1 as adult Low Responders [$p>0.05$], but were significantly faster to habituate than adult High Responders [$p=0.003$] (adult High- and Low-Responders did not significantly differ from each other [$p>0.05$]). Alternately, in OF3, late adolescents, adult High Responders, and adult Low Responders all took similar amounts of time to reach the habituation criterion [all $p>0.05$]. Together, these data suggest that there could be underlying neurobiological mechanisms for the attribute of locomotor response to a novel environment that are following a developmental trajectory, and they reach adult-like states by late-adolescence.

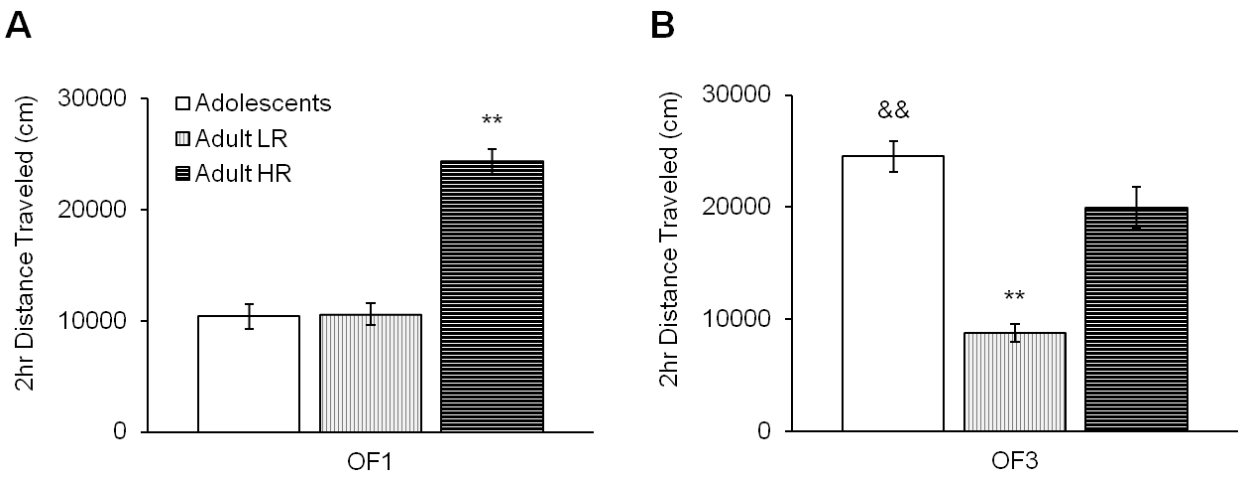


Figure 4.6. Early-adolescent distance traveled in OF1 resembles adult Low Responders, and late-adolescent distance traveled in OF3 resembles adult High Responders.

Adult rats (n = 24) were separated into tertiles based on total distance traveled during an initial Open Field (OF1). Because adolescent rats (n = 24; white bars) did not have significant correlations of distance traveled from OF1 to OF3, no group divisions were made. A two-way ANOVA with group (adolescents, adult High Responders, adult Low Responders) and test (OF1, OF3) as between-subjects factors revealed a significant test x group interaction [$p<0.001$]. A) In OF1, total distance traveled by early adolescents was significantly lower than total distance traveled by adult High Responders (n = 8; dark striped bars). B) In another Open Field exposure 5 weeks (adolescents) or 8 weeks (adults) after the original test (OF3), total distance traveled by late-adolescents was significantly higher than total distance traveled by adult Low Responders (n = 8; light grey striped bars). Additionally, while adolescent rats significantly increased total distance traveled from OF1 to OF3, adult Low Responders had similar levels of total distance traveled in both OF1 and OF3, and adult High Responders had similar levels of total distance traveled in OF1 and OF3. Values show group means ± SEMs. **$p\leq0.01$ vs. adolescent group; &&$p\leq0.01$ vs. OF1.

Table 4.1. Time to habituate locomotor activity in an Open Field in early adolescents is similar to adult Low Responders.

Time for distance traveled to reach habituation level criterion (see text for operational definition) was similar in early adolescents (n = 24) and adult Low Responders (n = 8) in the first exposure to the Open Field (OF1) [$p>0.05$], while adult High Responders took significantly longer to habituate compared to the adolescent group. Adult High- and Low-Responders did not differ from each other in time to habituate [$p>0.05$]. In a later exposure to the Open Field (OF3) 5 weeks (adolescents) or 8 weeks (adults) later, all three groups had similar habituation times [all $p>0.05$]. Values show means ± SEMs. **$p<0.01$ vs. adolescents.

Test	Group	Time to Habituate
OF1	**Adolescents**	13.8 (0.9)
	Adult LR	18.8 (1.8)
	Adult HR	**21.9 (2.3)****
OF3	**Adolescents**	18.8 (1.6)
	Adult LR	16.9 (1.3)
	Adult HR	20.6 (1.5)

4.4 DISCUSSION

The importance of understanding variations in individual response strategies in order to identify individuals most at risk for, or most resistant to, neuropsychiatric disorders is increasingly being appreciated. One behavioral attribute that varies greatly between individuals and has implications in both mood disorders and addiction is novelty-seeking (Bardo et al., 1996; Calvo et al., 2011; Dawe et al., 2004; Klebaur et al., 2001; Stedenfeld et al., 2011). Locomotor activity in response to a novel environment is considered to be an assay for novelty-seeking behavior in rodents [e.g.: (Calvo et al., 2011; Dellu et al., 1996; Jama et al., 2008; Kabbaj et al., 2000)]. Surprisingly, only a few have examined whether relative levels of distance traveled remain stable over time in individual rats. Furthermore, most studies that examine the relation between a behavioral attribute such as locomotor activity in a novel environment and the likelihood of exhibiting symptoms of neuropsychiatric disorders study this relation only in adults. Adolescence is a period characterized by increased risk-taking, and adolescents seem to be more responsive to novel stimuli (Doremus-Fitzwater et al., 2010; Douglas et al., 2003; McCormick & Mathews, 2010; Spear, 2000; Stansfield et al., 2004). We therefore aimed to investigate whether locomotor response to a novel environment was a stable attribute in both adult and adolescent male rats. To our knowledge, this is one of only a few studies to examine directly whether rankings of total distance traveled in a novel environment (2hr Open Field test) in outbred adult and adolescent rats are stable over time. It is also one of only a few studies to examine individual differences in locomotor responding to novelty in adolescent rats [but see (Arenas et al., 2014; Clinton et al., 2008; Mathews et al., 2010)].

We found that adolescent rats vary in the level of locomotor activity in a novel environment, similar to adult rats, with the most active rats traveling about 2.5-fold more than

the least active rats. However, different from adult rats, we found that an individual adolescents' relative ranking in locomotor activity differed markedly across repeated Open Field tests. That is, an adolescent rat that traveled a relatively shorter distance during one test may have been one of the most active rats during a subsequent test, and vice versa. These results demonstrate that locomotor response to novelty is not yet a stable attribute at this age. In contrast, adults' rankings in locomotor activity were quite stable over time. Adult rats classified as High Responders or Low Responders based on their total distance traveled in an initial 2hr test retained the same High/Low-Responder classification in the subsequent tests. Therefore, it is appropriate to consider the High/Low-Responder distinction a stable attribute in adult, but not adolescent, rats.

Interestingly, rankings of the locomotor response to a novel environment do appear to become stable by late-adolescence. We examined total distance traveled in the Open Field in adolescent rats that were part of a different study, and found significant correlations between two tests conducted in late-adolescence. Male rats (n = 12) were given an Open Field test on p24 (early adolescence), and two additional Open Field tests in late adolescence (p58 and p59). We replicated our findings of no correlation between the early- and late-adolescent total distance traveled [p24 vs. p58, $p>0.05$] (Figure 4.7A). In contrast to the lack of correlation between early and late adolescence, total distance traveled during the two tests in late-adolescence (p58 and p59) did correlate significantly [$r = 0.88$, $p<0.001$] (Figure 4.7B). The emerging correlation of distance traveled between two tests conducted in late adolescence again suggests that there is a developmental aspect to the relative level of locomotor responding in a novel environment.

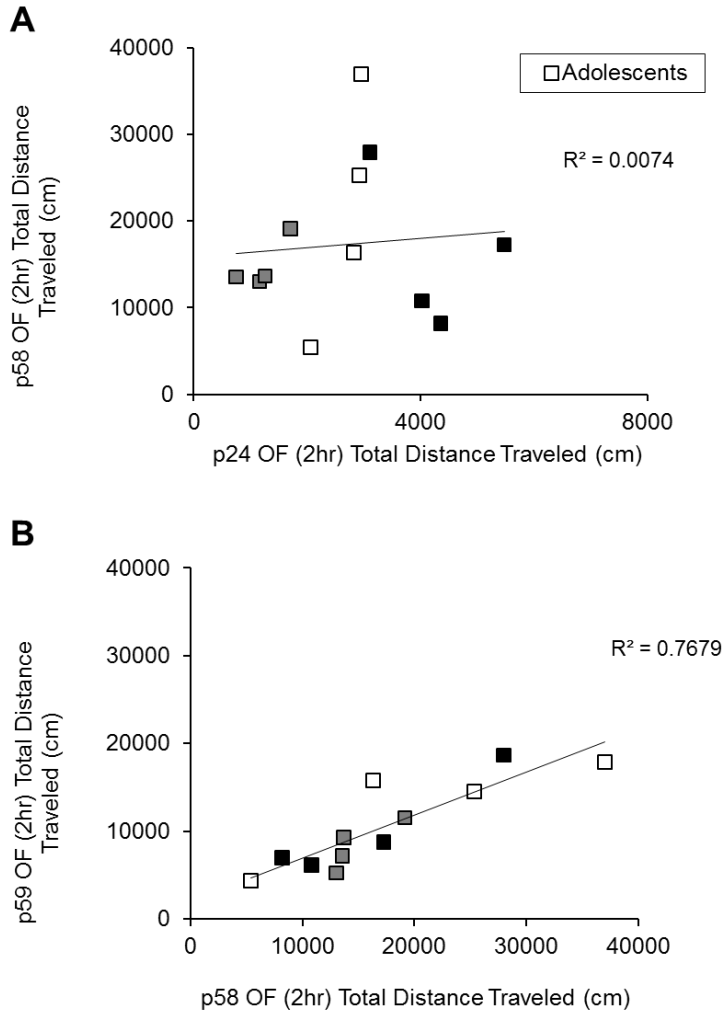


Figure 4.7. Late-adolescent Open Field locomotor activity is highly correlated.

In a separate experiment using adolescent Controls (exposed to equivalent conditions as those discussed in **Chapter 4**), multiple Open Field tests were given to adolescent male rats (n = 12). The first Open Field was given on postnatal day 24 (p24); rats were left relatively undisturbed in home cages for 5 weeks and were re-tested again in the Open Field on p58, and a final time on p59. A) When distance traveled was ranked into tertiles based on activity in the Open Field test given on p24, High Responders (top 1/3 of activity; black squares) and Low Responders (bottom 1/3 of activity; grey squares) did not remain consistent over time. Total distance traveled in p24 Open Field was not correlated with total distance traveled in p58 Open Field [$r = 0.09$, $p>0.05$]. B) Total distance traveled in p58 Open Field was significantly correlated with total distance traveled in p59 Open Field [$r = 0.88$, $p<0.001$]. Graphs show individual data points and regression lines/equations for all rats exposed to each test.

Although we did confirm that the locomotor response to (i.e., distance traveled in) a novel environment is a stable behavioral attribute, or "trait," in adult rats, the utility of this measure and what behavioral dimension it actually captures is open for debate. Many investigators use the High/Low-Responder classification when discussing distinctions in *novelty-seeking* behaviors [e.g.: (Calvo et al., 2011; Clinton et al., 2011; Dellu et al., 1996; Jama et al., 2008; Kabbaj et al., 2000); reviewed in: (Flagel et al., 2014)]. However, other data analyses yielded no correlations between multiple experimental variables related to novelty- and risk-seeking, including novelty place preference and numerous aspects of adult locomotor activity data (e.g., total distance traveled, slope and decay of curve-fits to individual locomotor activity profiles) [M. Schaff, E. Donny, A. Sved, unpublished observations]. Additionally, in the small group of late-adolescent animals that showed correlations between two Open Field tests at age p58 and p59 (discussed above), when we divided the group into High- and Low-Responders based on total distance traveled in p58, there were no significant differences between High- and Low-Responders on measures of anxiety-like behavior on the Elevated Plus Maze [all $p>0.05$] (Appendix Table A.2). Our finding of no group differences in anxiety-like behavior on the Elevated Plus Maze is in contrast to some of the original findings in High/Low- Responder adults that reported lower levels of anxiety-like behaviors in the Elevated Plus Maze in High Responder rats (Dellu et al., 1996; Kabbaj et al., 2000). Finally, with regard to the actual Open Field test, a long exposure to a novel environment allows for assessment of multiple aspects: the overall decline in locomotor response (as discussed above), and the more rapid habituation to the environment (Arenas et al., 2014; Mathews et al., 2010; Philpot & Wecker, 2008). Here, we found that adult rats took longer to reach the habituation criterion than did early adolescent rats, which suggests that that it took longer for the adults to become habituated to the novel

environment. Notably, when we compared adolescents with adults separated into High- and Low-Responder groups, adolescents habituated to the novel environment at a similar rate as Low Responder adults. High Responder adults took significantly longer to habituate to the Open Field than did the adolescents. However, when we examined whether time to habituate was correlated with total distance traveled in the adult High- and Low-Responders, it was not. Hence, a more pure measure of novelty-seeking, i.e., time to habituate to a novel environment, no longer completely overlapped with the High/Low-Responder attribute.

4.4.1 Conclusions

Here, we provide critical information confirming that locomotor response to novelty is a stable attribute in adult male rats. We also demonstrate that locomotor response to novelty is not a stable attribute in adolescent male rats. Adolescence is a known period of transition, with substantial maturation of reward circuitry (Andersen et al., 1997; Ernst et al., 2009; Laviola et al., 2003; Spear, 2000). Thus, it may be difficult to capture a true "trait" of novelty-seeking at this age, because the neurobiological systems underlying novelty-seeking may not be yet mature. We saw a group-wide shift in distance traveled from early- to late- adolescence, with activity similar to adult Low Responder behavior in early-, and activity similar to High Responder behavior in late-adolescence. This group-wide shift in activity profile could mean that the attribute of locomotor response to novelty develops during the course of adolescence. It remains to be determined if *novelty-seeking* per se is the adult attribute being assessed with the 2hr Open Field test, and whether it can capture a developmental shift from early adolescence into late adolescence and adulthood.

5.0 GENERAL DISCUSSION

5.1 SUMMARY OF FINDINGS

Stress is a precipitating factor for many neurological and psychiatric disorders. The nature and degree of stress, age at stress exposure, and individual vulnerability are all critical factors that determine consequential outcomes of stress. Although much is known about the long-term effects of adolescent stress exposure, a key area lacking in substantial information is that of understanding the immediate effects of unpredictable chronic mild stress *during adolescence*. Because adolescence is a time of behavioral, physiological, and neural transition, it is important to know whether stress during adolescence shifts functioning immediately, or if prolonged changes observed later in life are a result of protracted change that continues after the adolescent stress exposure ends (Figure 5.1). Understanding whether stress during adolescence results in immediate, delayed, or an absence of depression has important implications for our understanding of the relation between brain circuitry and function, and susceptibility to depression: if adolescents are more vulnerable to stress-induced depression, then it could tell us that the characteristics of an adolescent brain endow increased susceptibility to stress-induced depression. Alternatively, if adolescents are less susceptible, it could tell us that characteristics in relevant brain regions prevalent in the adolescent brain may endow resilience to stress-induced depression. Additionally, this work could inform us on prevention and treatment – if stress shifts

functioning immediately, it would be critical to implement treatments right away for best alleviation of detrimental outcomes. If, however, stress during adolescence initiates a series of changes that results in symptom emergence later in life, it would be important to explore the mechanisms that underlie those changes, because they may shed light on mechanisms that underlie stress-induced depression. It would also offer the opportunity to determine whether steps could be taken to prevent or blunt symptom development. Finally, if the outcome of stress during adolescence is different to what is observed after stress later in life, it would be pertinent to know: 1) what is unique about the adolescent stress system that yields a different outcome; and 2) how the adolescent stress outcome shifts to the adult "endpoint," in order to identify means of preventing detrimental outcomes while possibly enhancing positive outcomes.

The goals of the present studies were to determine the immediate effects of unpredictable chronic mild stress exposure during adolescence (**Chapter 2**); and to evaluate which aspects, if any, could be used as predictors (environmental or inherent characteristics) for determining individuals most at-risk for detrimental effects of adolescent chronic stress exposure (**Chapter 3 and Chapter 4**). In the following sections we will review the findings of the experimental chapters, and then discuss the broader implications of these findings in the context of chronic mild stress in adolescence, the issue of peri-weaning shipping experience, and the issue of examining novelty-seeking as an attribute. We will also discuss how these experimental findings serve as critical groundwork for future studies on understanding the long-term effects of chronic stress in adolescence.

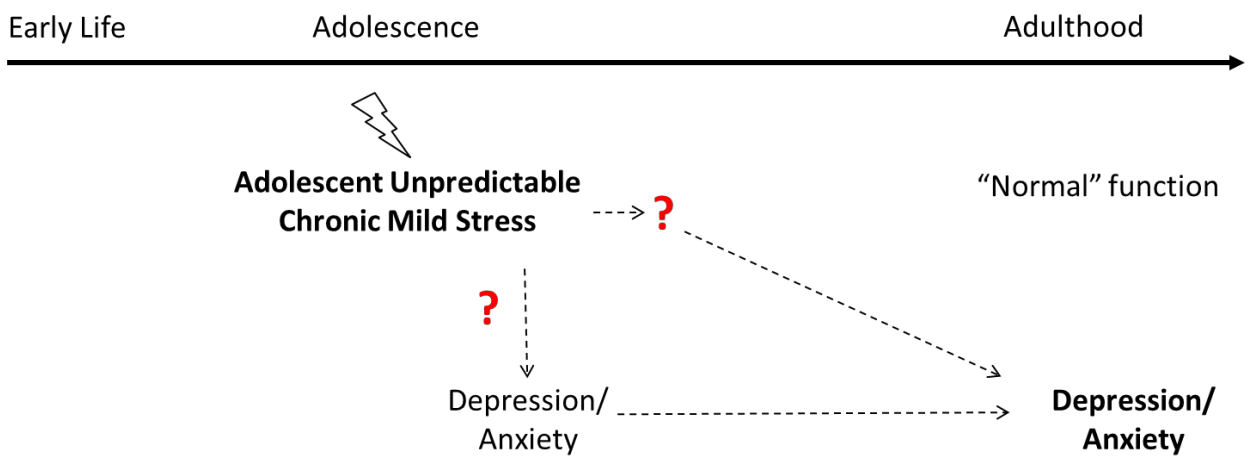


Figure 5.1. Does UCMS during adolescence cause depressive- and anxiety-like symptoms in adolescents?

Diagram of central question. It is known that unpredictable chronic mild stress (UCMS) during adulthood can cause depressive- and anxiety-like symptoms in adults. It is also known that chronic stress during adolescence can lead to depressive- and anxiety-like-symptoms in adulthood. What is not known is whether adolescent UCMS exposure causes symptoms that occur right away, or if adolescent UCMS exposure shifts the trajectory of on-going brain development, and depressive- and anxiety-like symptoms only are manifested later in adulthood.

5.2 STUDYING DEPRESSION IN ADOLESCENCE

The principal goal of this thesis was to study depression in adolescence. Adolescence is a time of great biological, psychological, and social transition; and numerous neuropsychiatric disorders begin to arise in adolescence, or develop as a direct result of adolescent experiences (Oldehinkel & Bouma, 2011; Spear, 2000). Estimated lifetime prevalence for adolescent Major Depressive Disorder ranges from 4-24% (depending on diagnostic criteria and study sampling) (Angold & Worthman, 1993; Oldehinkel & Bouma, 2011), and the Centers for Disease Control and Prevention list suicide as the third leading cause of death among people 10-24 years old (Centers for Disease Control and Prevention, 2010). Although depressed adolescents can have similar symptoms as depressed adults (e.g., anhedonia, hypersomnia, impaired concentration), the underlying neurobiological changes and treatment responses are often different (Andersen & Teicher, 2008; Fleming & Offord, 1990). For example, depressed youth fail to respond to tricyclic antidepressants (Birmaher et al., 1996; Hazell et al., 1995; Kaufman et al., 2001). We chose to use unpredictable chronic mild stress (UCMS) to induce depressive-like symptoms because this paradigm is viewed as a well-validated model of stress-induced depression for adults that offers face, construct, and predictive validity (Willner, 1997b, 2005). We examined the effects of UCMS during adolescence on adolescent behavior, neuroendocrine, and neural factors in **Chapter 2**, and predicted that we would observe robust effects of UCMS in the adolescents. This prediction was based on demonstrations that adolescents are more vulnerable to environmental and experiential factors [e.g.: (Doremus-Fitzwater et al., 2010; Heim & Nemeroff, 2001; Lupien et al., 2009; McCormick & Mathews, 2007, 2010; Vazquez, 1998)]. Different from expectation, we found that UCMS exposure causes a very different behavioral, neuroendocrine, and neural profile than what is typically observed in adults.

The effects of UCMS in adult rodents have been well-documented [e.g.: (Bergström et al., 2008; Bielajew et al., 2002; Grippo et al., 2003; Grippo et al., 2005a; Grønli et al., 2006; Li et al., 2009; Willner, 1997b, 2005)]. In adult rats and mice, UCMS blunts weight gain, and induces the key symptom of depression, anhedonia, as assessed by the sucrose preference test (Ayensu et al., 1995; Bielajew et al., 2002; Grippo et al., 2005b; Grønli et al., 2004). Adult rodents also demonstrate anxiety-like behaviors, as assessed by the Elevated Plus Maze and the Open Field test (Lupien et al., 2009; Willner, 1997b, 2005). Additionally, UCMS in adulthood was demonstrated to produce various neuroendocrine effects, including increased plasma corticosterone levels, and decreased negative feedback onto the hypothalamic-pituitary-adrenal (HPA) axis, as assessed by the dexamethasone suppression test (Ayensu et al., 1995; Bielajew et al., 2002; Grippo et al., 2005a; Helmreich et al., 2008). Finally, many investigators have demonstrated a downregulation of hippocampal glucocorticoid receptors, and a downregulation of the transcription factor cyclic-AMP response element-binding protein (CREB) in the hippocampus after UCMS in adulthood (Adzic et al., 2009; Guidotti et al., 2012; Muschamp et al., 2011; Nestler et al., 2002).

Our studies yielded no evidence for depressive-like neurobiological changes in adolescent rats exposed to UCMS. There were no changes in hippocampal glucocorticoid receptor levels. Additionally, levels of a transcription factor implicated in emotional regulation, CREB, were not different between controls or UCMS-exposed adolescents in multiple limbic regions. These neurobiological findings are consistent with our behavioral findings of a lack of depressive-like behaviors in adolescents exposed to UCMS. At the same time, we identified neurobiological changes in adolescent rats exposed to UCMS that, together with the observed blunted corticosterone levels, suggested there may be a *protective* effect via blunting of an acute

stress response. There was a trend for a lower phosphorylation state of hippocampal glucocorticoid receptors in adolescents exposed to UCMS versus controls. Based on evidence that lower levels of glucocorticoid receptor phosphorylation can correspond with lower glucocorticoid receptor transcriptional activation and decreased expression of glucocorticoid receptor-responsive genes (Blind & Garabedian, 2008; Chen et al., 2008), this again suggests blunted activation of the stress system. We also observed blunted levels of phosphorylated CREB in the paraventricular nucleus (PVN) after an acute stress in adolescents exposed to UCMS. In the PVN, CREB is involved in transcription of corticotrophin-releasing factor, while also being subject to phosphorylation by this hormone (Liu et al., 2011; Seasholtz et al., 1988). Thus, the attenuation of acute stress-evoked phosphorylated CREB suggests less impact by the acute stressor in the UCMS-exposed adolescents.

To our knowledge, there is one study that used almost identical stressors as employed in our paradigm (Toth et al., 2008). Toth and colleagues exposed adolescent rats to UCMS for 4 weeks starting on postnatal day 30 (2008). Toth et al. found no evidence for reduction in sucrose preference, and no difference in immobility in the forced swim test between UCMS-exposed rats and controls. Thus, our findings of a lack of depressive-like behaviors after UCMS during adolescence agree with the findings of Toth and colleagues (2008). Surprisingly, Toth et al. found that circulating corticosterone levels are significantly elevated relative to control levels after 2 weeks of UCMS. However, 2 weeks *after the completion* of UCMS, circulating corticosterone levels of UCMS-exposed adolescents were significantly *lower* than those of control rats (Toth et al., 2008). We also found that, after a total of 5 weeks of UCMS, corticosterone levels were significantly *lower* in UCMS-exposed adolescents than in controls. The data of Toth et al. (2008), combined with our results, raise the intriguing idea that UCMS

initially induces an increase in corticosterone levels, but during the course of the UCMS, something occurs to decrease corticosterone levels. The function of the HPA axis and subsequent glucocorticoid release can be influenced by glucocorticoid receptor function [reviewed in: (Pariante, 2006)]. As aforementioned, we saw no change in overall levels of glucocorticoid receptors in the hippocampus, and no major shift in phosphorylation state of glucocorticoid receptors in limbic circuitry. It could be that reduced corticosterone levels reflect a shift in glucocorticoid receptor localization within the cell, namely, a shift from nuclear to cytoplasmic localization (Adzic et al., 2009). Thus, changes in glucocorticoid receptor trafficking could result from UCMS exposure in adolescence, which, in turn, alters circulating glucocorticoid levels. One potential key player in these adaptations is part of a chaperone protein complex that binds the glucocorticoid receptor to keep it outside of the nucleus: FK506 binding protein 51 (FKBP5) (Finsterwald & Alberini, 2014; Guidotti et al., 2012; Pérez-Ortiz et al., 2013).

It may be the case that the "threshold" level of stress required to induce depressive-like behaviors is shifted in adolescents compared to adults (Figure 5.2). Indeed, other paradigms of chronic stress in adolescence have also revealed a lack of anxiety- or depressive-like behaviors in adolescent rats. For example, males exposed to social stress (daily 1hr isolation and new cage partner) in mid-adolescence (p30-45) showed no differences in anxiety-like behavior on the Elevated Plus Maze compared to controls (McCormick et al., 2008). A study using chronic variable stress (which includes stronger stressors, such as restraint and cold stress) showed mixed results, with adolescents exposed to stress from p30-45 showing no difference in circulating corticosterone levels and no evidence of behavioral despair in the forced swim test compared to controls; but adolescents exposed in late adolescence (p50-64) showed exaggerated somatic effects and increased corticosterone levels (Jankord et al., 2011). Also, exposure to social defeat

in early adolescence induced proactive behaviors in a defensive-burying task and forced swim test (suggesting less of a detrimental effect) (Bingham et al., 2011). Finally, researchers consistently have demonstrated depressive-like behaviors in adult rats exposed to corticosterone in the drinking water (Gourley & Taylor, 2009; Gourley et al., 2008a; Gourley et al., 2008b); however, Torregrossa and colleagues have been unable to find evidence of anxiety- or depressive-like behaviors when using the same protocol in adolescent rats [M. Torregrossa, personal communication]. It could be, then, that an additional stress, or very severe stress, is needed to induce depressive-like phenotypes in adolescence.

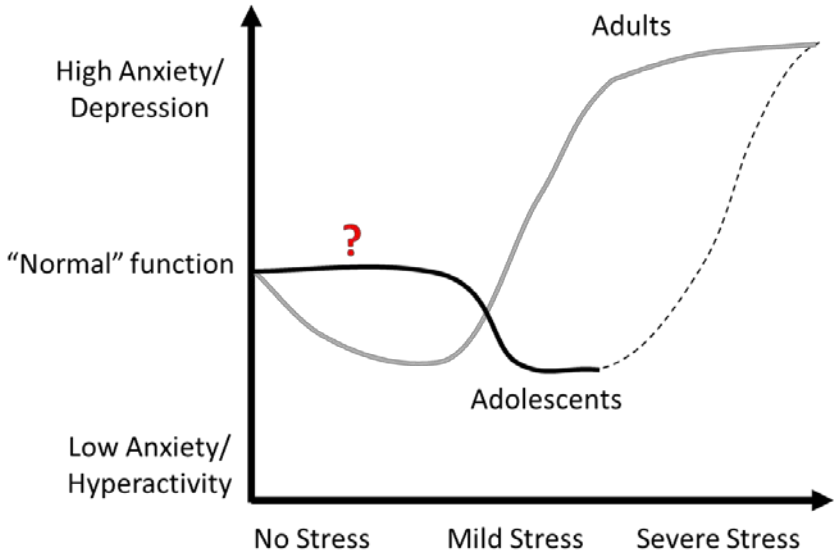


Figure 5.2. Do adolescents have a different threshold for the effects of UCMS?

It is well-established in the literature that exposure to a relatively mild stress paradigm, unpredictable chronic mild stress (UCMS), causes adult rats to exhibit depressive- and anxiety-like symptoms. It has also been shown that chronic stress in adolescence can cause depressive- and anxiety-like symptoms in adulthood. Our study shows that UCMS during adolescence is not sufficient to induce depressive- or anxiety-like symptoms. Instead, it causes adolescent rats to become *hyperactive* and *less anxious*. This outcome could mean that adolescents are on a different trajectory than adults after UCMS exposure. Stress exposure may need to be more severe in adolescence, or adolescents may need to encounter a second episode of stress exposure, in order to push them on a trajectory towards depressive- and anxiety-like symptoms manifested in adulthood.

5.3 NEUROBIOLOGICAL MECHANISMS OF STRESS DURING ADOLESCENCE

We examined a number of stress- and emotion-related proteins in the brains of adolescent rats exposed to UCMS versus control conditions: glucocorticoid receptors, CREB, and extracellular signal-regulated kinase1/2 (ERK). Despite a lack of robust differences for individual proteins, the combination of results may inform on the neurobiological mechanisms altered after chronic stress during adolescence. Glucocorticoid receptors, CREB, and ERK are expressed in limbic, stress, and reward circuitry, including the prefrontal cortex, the hippocampus, and the striatum (Carlezon Jr et al., 2005; McKlveen et al., 2013; Nestler et al., 2002; Russo & Nestler, 2013). All three proteins could participate in the same signaling loop. The intracellular ERK signaling cascade can be influenced by glucocorticoid exposure (Gourley et al., 2008a; 2008b), and CREB is a downstream target of ERK [reviewed in: (Carlezon Jr et al., 2005; Shaywitz & Greenberg, 1999; Thomas & Huganir, 2004)]. Finally, glucocorticoid production can be CREB-dependent, and glucocorticoids themselves can stimulate or inhibit CREB phosphorylation and, thus, its transcription-regulatory potential [reviewed in: (Finsterwald & Alberini, 2014; Kovacs, 2013; Legradi et al., 1997; Stern et al., 2011)]. Our findings suggest that UCMS during adolescence shifts neurobiological functioning towards a lower stress-reactive state, where phosphorylated CREB and circulating glucocorticoid levels are lower after an acute stress.

5.4 WHAT HAPPENS FROM ADOLESCENCE TO ADULTHOOD?

Studies investigating the effects of chronic stress during adolescence on functioning in adulthood also have yielded fairly consistent results, in that rats with a history of chronic stress exposure in adolescence were found to exhibit depressive- and enhanced anxiety-like symptoms in adulthood [e.g.: (Green et al., 2013; McCormick et al., 2008)]; this is true for a variety of stress paradigms (e.g., social instability, UCMS, chronic variable stress). What is less well-known is whether chronic mild stress during adolescence causes depressive- and anxiety-like symptoms *in adolescence*. Contrary to expectation, we found that UCMS during adolescence *does not* cause anhedonia or an increase in anxiety. Instead, we found that UCMS during adolescence causes hyperactivity, decreased anxiety-like behaviors, lower levels of basal plasma corticosterone, and a pattern of neurobiological results consistent with the absence of depressive-like and stress-reactive symptoms in adolescent rats.

Our findings, in conjunction with what has been shown regarding stress during adolescence inducing adult depressive- and anxiety-like symptoms, raise exciting new questions for future investigation (Figure 5.3). First, it is imperative to conduct a study in which adolescent rats are exposed to UCMS during adolescence, allowed to age to adulthood, and re-examined for behavioral, neuroendocrine, and neurobiological changes. We know that adolescents are hyperactive and less anxious after UCMS exposure in adolescence. What we do not yet know is whether the hyperactivity and decreased anxiety observed here is a permanent shift in behavioral profile, and these behaviors persist into adulthood. We also do not know if stress exposure during adolescence causes shifts in the developmental trajectory of maturing brain systems to yield depressive-like and high anxiety-like behaviors in adulthood. Indeed, it was demonstrated that stress manipulations in adolescence ultimately end in adult depression and anxiety (as

discussed above). However, those studies do not report on the behavioral or brain state of the adolescent animals. Understanding the developmental shift of brain systems and the consequences of deviating from the normal biological trajectory will aid in understanding the relation between brain and behavior as it pertains to disease. For example, if deviations in brain system trajectories sets up vulnerability in mood- or reward-related systems, closer study of those brain alterations may be warranted to understand the mechanistic etiology of depression. Identifying whether hyperactivity and low anxiety/high risk-seeking behaviors in adolescence after mild stress persist into adulthood, or instead shift to depression- and anxiety-like phenotypes, is also important for informing on treatment/prevention methods. If hyperactivity after mild stress in adolescence is shown to be the manifestation of what will become depression and anxiety in adulthood, then preventative treatments should be started in adolescence. If, alternatively, hyperactivity after mild stress in adolescence suggests a more resilient adulthood, then measures to ensure this trajectory is maintained would be beneficial. We also acknowledge that there may be sex differences. Mood disorders are known to be more prevalent in adult women and in post-pubertal girls; and human and clinical work suggest that the HPA axis can be affected by female sex hormones [(Bangasser et al., 2010; McCormick & Mathews, 2007); reviewed in: (Andersen, 2003; Oldehinkel & Bouma, 2011)]. Thus, it would be pertinent to re-examine the proposed experimental questions using females as well as males.

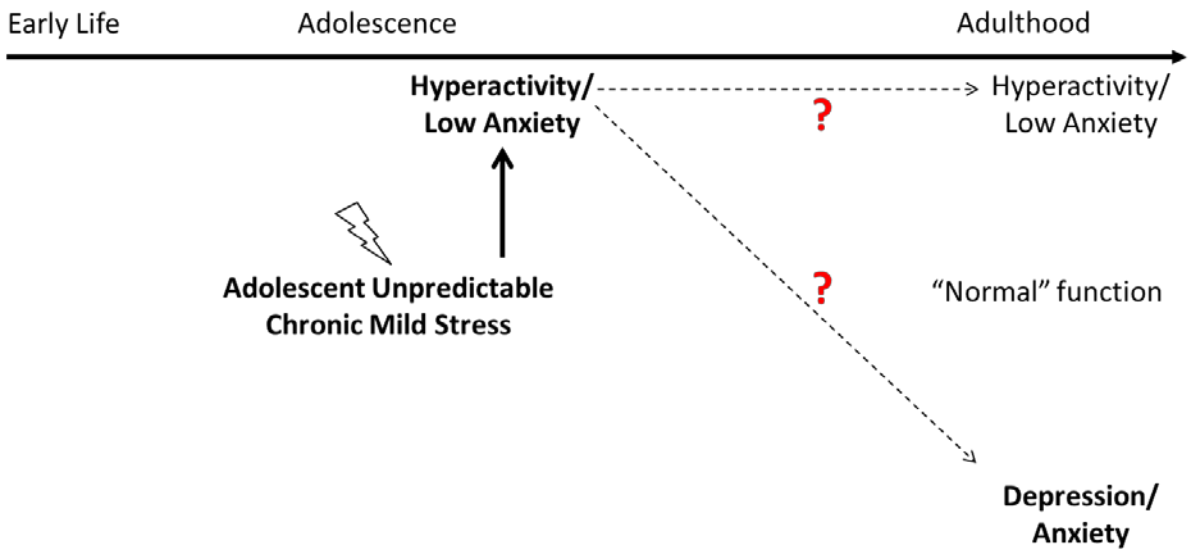


Figure 5.3. What does adolescent UCMS-induced hyperactivity/low-anxiety translate to in adulthood?

Diagram of future questions. We found that UCMS during adolescence caused robust hyperactivity and low anxiety-like behaviors. Others have shown chronic stress in adolescence to cause depressive- and anxiety-like symptoms in adulthood. What is currently unknown is whether the observed UCMS-induced hyperactivity/low anxiety in adolescents in the current study has shifted the trajectory of ongoing brain development. It could be that adolescents with mild stress exposure (as seen here) are protected from developing depressive- and anxiety-like symptoms later in adulthood. Alternatively, it could be that, although adolescents appear to have shifted trajectories, the adulthood end-point will still result in depressive- and anxiety-like symptoms. Thus, it could be that the symptoms manifested in adolescence are different from what will be seen later in adulthood.

5.5 PERI-WEANING SHIPPING EXPERIENCE MATTERS

Investigators who do not have access to in-house breeding colonies have their research animals shipped to their research facility. During the process of shipping, rodents are likely exposed to a variety of stimuli that often are used as discrete stressors: noise, vibrations, changes in temperature, and noxious odors are just a few (Laroche et al., 2009). In fact, studies have demonstrated detrimental effects of shipping on rodents: upon arrival, shipped rodents exhibited increased circulating corticosterone levels that took 48hr to recover (Landi et al., 1982); and male and female mice shipped during the peripubertal age (p42) demonstrated reduced hormone-induced sexual behavior in adulthood (Laroche et al., 2009). Thus, shipping itself is stressful to rodents, and exposure to stress early in life can have drastic influence on behavioral and endocrine stress responses (Levine, 2005). Because rodent adolescence (p28-59) is limited to just a few weeks (McCormick & Mathews, 2007; Tirelli et al., 2003), adolescent experimental manipulations often require animals to be shipped close in time to weaning age (peri-weaning) (Eiland & Romeo, 2013) if no in-house breeding colony is available. Weaning young rodents, i.e., separation from a lactating dam, is naturally a gradual transition and has been observed to occur until as late as p34 in rats (Cramer et al., 1990; Galef, 1981; Thiels et al., 1990; 1988). Experimentally, weaning usually occurs on p21 (Adriani & Laviola, 2002), and can be stressful to the young rodents: studies have reported that rodents weaned early (p14-16) demonstrate increased anxiety-like behaviors and increased circulating corticosterone levels at adulthood, relative to rodents weaned later (p30) (Ito et al., 2006; Kanari et al., 2005).

Aside from the potential immediate detrimental effects of the early-life stresses of weaning and shipping previously discussed, there are potential detrimental effects later in life as well. Akin to what some term a “two-hit hypothesis,” we believe that the peri-weaning

experience of adolescent rats could serve as the “first-hit” in creating a stress-vulnerable animal. Two-hit models exist for complex diseases, including cancer and schizophrenia. In the case of schizophrenia, a two-hit hypothesis posits that early life genetic or environmental factors interfere with development of the central nervous system, which produces vulnerability to psychiatric disorder. However, the onset of symptoms does not occur until a subsequent “hit” from additional environmental factors, such as stress, later in life [reviewed in: (Maynard et al., 2001)]. Applying this concept to the aforementioned peri-weaning concern, it is possible that the combination of weaning and shipping on p21 is detrimental enough to alter developmental trajectories and induce psychiatric vulnerabilities, putting adolescent animals at heightened risk, prior to any experimental manipulations at all. Indeed, the most profound UCMS effect in the current set of studies was observed in animals experiencing the stress of weaning and shipping concurrently (**Chapter 2; Chapter 3, Table 3.3**). Despite a different pattern of results than what is observed in adults exposed to UCMS (discussed above), adolescent rats that were weaned and shipped concurrently exhibited clear hyperactivity and decreased anxiety phenotypes after exposure to UCMS. However, our findings described in **Chapter 3** suggest that the relationship between peri-weaning conditions and behavior may not be that straightforward. Pre-weaning rats that were shipped with a dam experienced conditions that were sufficient to evoke behavioral signs of hyperactivity early in life: Open Field activity of ship-with-dam rats was significantly elevated relative to that of rats born in-house (**Figure 3.8**); and, relative to control rats weaned and shipped on p21, rats shipped with a dam exhibited some signs of decreased anxiety in the elevated plus maze in late adolescence regardless of whether or not they were exposed to UCMS (**Figure 3.6A**). Our findings show that 1) the two groups exposed to UCMS exhibited overall similar behavioral profiles, and 2) the Open Field activity level in early adolescence, before

exposure to stress, by rats weaned and shipped on p21 was comparable to rats born in-house. Taken together, our findings suggest that the effect of weaning and shipping on p21 may not be overly-harmful. Two additional studies are needed in order to gain a better understanding of the effects of the peri-weaning shipping experience: 1) a group of rats born in-house (with no shipping stress) should be exposed to the same UCMS protocol as used in the current work during adolescence, and tested in late adolescence (to match the current set of studies); and 2) rats of all three conditions exposed to UCMS or control conditions during adolescence should be allowed to age to adulthood in order to assess long-term behavioral and neurobiological changes. Our work highlights that peri-weaning experience does matter for studies utilizing adolescent rodents.

5.6 INDIVIDUAL DIFFERENCES IN LOCOMOTOR ACTIVITY

Although no neuropsychiatric disorder can be completely explained by one simple characteristic, animal models have been used in an attempt to identify key neurobiological features underlying a broad attribute, or "trait," that could be related to the disorders. For example, animal models of individual differences in attributes related to novelty- and sensation-seeking are used in order to understand the neurobiology underlying increased vulnerability to addiction and anxiety/ depression [e.g.: (Belin et al., 2011; Flagel et al., 2010; Piazza et al., 1989; Stead et al., 2006)]. Two such examples are the High/Low-Responders and the Sign/Goal-trackers. Both models were developed to identify a specific trait, or attribute, that has been linked to increased propensity for drug addiction in humans [reviewed in: (Flagel et al., 2014; Robinson et al., 2014)]. In the High/Low-Responder model, an attempt is made to capture the human trait of novelty-seeking,

by examining rodents' locomotor activity in a novel environment (Open Field chamber) over a long period of time (1-2hr). Specifically, adult rodents that display very low levels of distance traveled in a novel environment over a 2hr Open Field test (Low Responders) were shown to be prone to develop anxiety- and depressive-like behavior after stress (Stedenfeld et al., 2011), whereas those that exhibit very high levels of distance traveled (High Responders) were shown to be prone to exhibit elevated corticosterone release in response to the novel environment, reduced inhibitory control, elevated risk-taking, and addictive-like behaviors (Belin et al., 2011; Calvo et al., 2011; Flagel et al., 2010; Stead et al., 2006; Stedenfeld et al., 2011).

In the Sign/Goal-tracker model, an attempt is made to capture differential tendencies to attend to cues that predict reward versus other external cues (i.e., differences in incentive salience of cues). In order to capture variations in propensity to attribute incentive motivational properties to cues, rats are tested in an appetitive Pavlovian conditioning paradigm. Briefly, rodents express a preference to approach either a discrete stimulus (cue) that has been paired with reward (i.e., Sign-trackers) or the location of food/reward delivery (i.e., Goal-trackers) [reviewed in: (Meyer et al., 2012; Robinson et al., 2014)]. Studies have implicated a risk for addiction in the Sign-tracker animals (Flagel et al., 2008; 2006; Uslaner et al., 2006). Sign-trackers were found to exhibit higher cue-induced responding for cocaine, worked harder to obtain cocaine, and had more robust cocaine-induced reinstatement of drug-seeking behavior compared to Goal-trackers (Saunders & Robinson, 2011; Yager & Robinson, 2012). Additionally, Sign-trackers also were shown to have elevated serum corticosterone levels during Pavlovian conditioning sessions, and higher levels of dopamine in the striatum after conditioning sessions (Flagel et al., 2011; Tomie et al., 2000).

Notably, one group examined Sign-tracking and Goal-tracking propensities in their bred-High/Low Responder rats, and reported an overlap of bred-High Responders with sign-tracking and bred-Low Responders with goal-tracking tendencies (Flagel et al., 2011; 2010). However, the bred-High/Low-Responder rats were tested on a few others tests of impulsivity, with mixed results: bred-High Responder rats were *less* impulsive than bred-Low Responder rats in a delay-discounting task, and there were no group differences in a probabilistic-choice task. Thus, there seem to be multiple attributes underlying the Goal-tracker/Sign-tracker domain, which may or may not completely overlap with attributes in High- and Low-Responders. Together, these varied findings in specific models of individual characteristics suggest that identifying behavioral attributes as a way to classify individuals most at risk for a disease may be less straight-forward than originally anticipated.

In **Chapter 4** we aimed to examine the stability of the High/Low-Responder attribute in both adult and adolescent rodents by assessing locomotor response to a novel environment in a 2hr Open Field Test, and re-assessing it later. Few studies re-test the same adult animal to confirm its original classification of High- or Low-Responder, and, to our knowledge, we may be the first group to examine the stability of this attribute in adolescent animals. We examined behavior in control animals only, and compared adolescents to adults that were classified as High- or Low-Responders. We originally aimed to identify adolescents most at risk towards the detrimental effects of UCMS, similar to what others have done in adult rats. However, prior to examining stress effects based on an attribute categorization of the adolescents, we wanted to confirm that the behavioral tendencies were a stable attribute. We confirmed that adult classifications into High-Responders (upper third of the distribution of total distance traveled) and Low-Responders (lower third of the distribution of total distance traveled) were highly

reliable, i.e., High-Responders remained High-Responders. Confirmation that locomotor response to a novel environment is a stable trait in adult rats is a critical contribution to the field.

Two points of concern arose from our analyses of locomotor activity in a novel environment in adolescents and adults and highlight the question of what actually is being captured with this measure. First, adolescent High/Low-Responder classifications were very inconsistent: adolescents did not maintain the same distributions of total distance traveled (High-Responders were *not* still High-Responders upon retest). The lack of stable rankings over time indicates that the attribute of locomotor response to novelty, as it is being captured, is not present in young adolescents. Second, because this test was designed to capture the behavioral response to a novel environment, it inherently measures locomotor habituation to that environment. We examined habituation directly by monitoring the time it took for the total distance traveled to be reduced below half the maximum distance traveled in the test. This measure could not significantly distinguish between adult High- and Low-Responders, and it was not correlated at all between successive Open Field tests. There were, however, similarities between early adolescents and adult Low-Responders, and late adolescents and adult High-Responders. The lack of a difference between adult High- and Low-Responder in the time required to habituate using the above measure raises a critical question of what the High/Low-Responder attribute, or "trait" in the case of the adults, actually encompasses – it is not a straight-forward concept, like habituation. Although we do not dispute that the High/Low-Responder classification has been useful in identifying individuals most at risk for depressive- and addictive-like outcomes, more studies in which multiple attributes are examined for the purpose of identifying behavioral clusters may be needed to gain a better understanding of what this frequently employed "trait" entails or captures.

5.7 CONCLUSIONS

The work presented in this thesis begins to fill a critical void in the field on what the effects of chronic mild stress during adolescence are *in adolescence*. We show that unpredictable chronic mild stress during adolescence is capable of inducing a behavioral, neuroendocrine, and neural profile characterized by hyperactivity, increased exploration and risk-seeking, and a reduced stress response in neuroendocrine and brain stress systems. An exciting new question arises from this work on whether chronic mild stress during adolescence yields a resilient or more vulnerable adult. It will be beneficial to understand whether the effects of chronic stress during adolescence shift behavioral, neuroendocrine, and neural set-points immediately, or whether the shifts occur over time between adolescence and adulthood. These results lay the groundwork for those important future studies.

Additionally, the work in this thesis highlights two important points about individual differences. First, in rodents, peri-weaning experience is a critical factor to be aware of when performing subsequent stress manipulations during adolescence. We present evidence that, although probably not as ideal as raising animals in-house, young male rats that have been weaned and shipped concurrently on p21 may not be profoundly impaired by the early stress exposure; but peri-weaning experience is a factor of which investigators must be cognizant as they conduct adolescent work. Second, we show here that locomotor response to novelty, i.e., activity in a novel Open Field Chamber over a 2hr test, is a stable attribute in adult male rats, but not in male adolescents.

Finally, we bring attention to the fact that it is unclear what “locomotor response to novelty” means in terms of the underlying trait. As novelty-seeking is thought to be a key characteristic of the period of adolescence in general, it is interesting to note that the 2hr Open

Field test does not actually highlight that attribute in a stable manner until the very end of adolescence. Together this work begins to highlight the effects of UCMS during adolescence, the contribution of differential peri-weaning experience to experimental outcome, and the importance of identifying what attributes are measured for in standard models of individual differences.

APPENDIX A

SUPPLEMENTAL INFORMATION FOR EXPERIMENTAL CHAPTERS

A.1 CHAPTER 2 SUPPLEMENTAL RESULTS

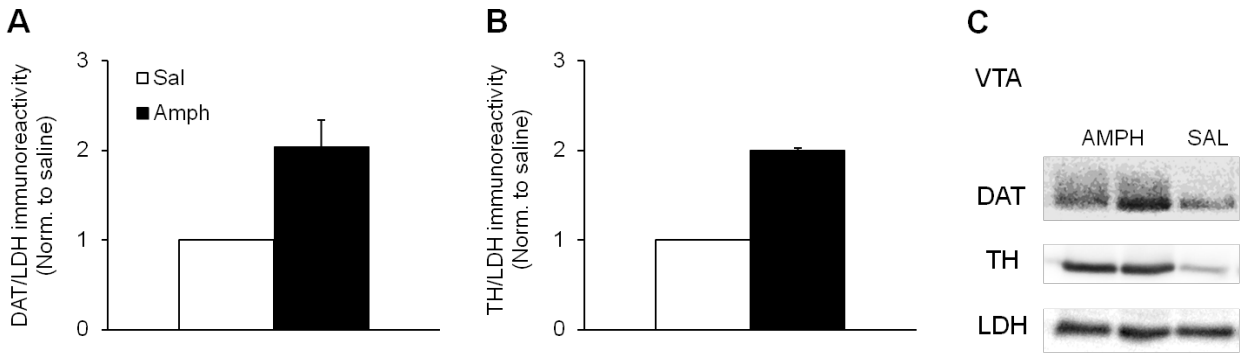


Figure A.1. Western blotting techniques for dopamine transporter and tyrosine hydroxylase can detect group differences.

We confirmed that our Western blotting protocol and antibodies used were able to detect group differences, to support the validity of the negative findings reported in **Chapter 2**. Adult Sprague-Dawley rats (n = 2) were administered intraperitoneal (i.p.) amphetamine (AMPH; 3mg/kg, dissolved in 0.9% saline) for 5 days, and then went through 2 weeks of withdrawal in the home cage. A control animal was given i.p. saline (Sal; 1ml/kg) for 5 days, and was left undisturbed in the home cage for 2 weeks. Rats were sacrificed and ventral tegmental area (VTA) tissue was collected and processed for Western blotting as described in **Chapter 2**. Graphs show quantification of: A) dopamine transporter (DAT) and B) tyrosine hydroxylase (TH) immunoreactivity, corrected by total protein (lactate dehydrogenase; LDH) immunoreactivity, normalized to saline levels. C) Representative blot of DAT, TH, and LDH in AMPH and Sal-treated rats.

Table A.1. Changes in weight gain are not correlated with hyperactivity in the Open Field.

Controls (n = 10) and UCMS (n = 10) were tested once in the Open Field prior to the start of UCMS (pre-stress) and again during the fifth week of UCMS (post-stress). The percent increase in locomotor activity for each rat was calculated as [(total distance traveled (cm) post-stress – total distance traveled (cm) pre-stress) / total distance traveled pre-stress] x 100. The percent increase in weight was calculated as [(final weight (g) – initial pre-stress weight (g)) / initial pre-stress weight] x 100. Individual rats' (n = 10) percent increase in weight were not correlated with percent increase in Open Field activity [$r^2 = 0.06$, $p>0.05$]. Similar results were found if the UCMS and Con groups were correlated separately [both $p>0.05$]. Values expressed are group means ±SEMs.

Group	%increase OF	%weight gain
Con	395 (79)	489 (23)
UCMS	805 (137)	422 (15)
Correlation	r2 = 0.06	p=0.29

A.2 CHAPTER 4 SUPPLEMENTAL RESULTS

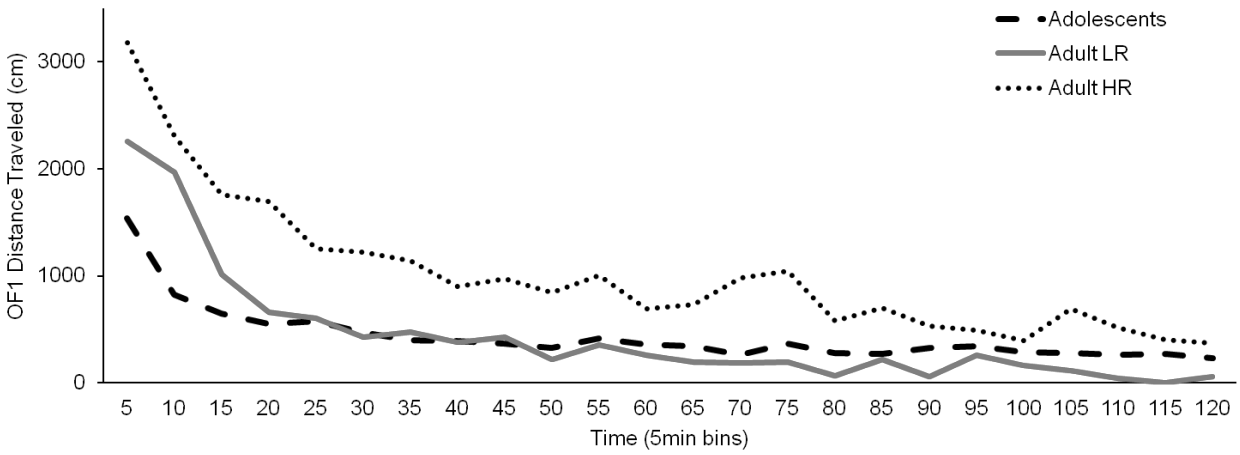


Figure A.2. Early-adolescent locomotor activity in a novel environment resembles adult Low Responder activity.

Locomotor activity in a novel environment was recorded for 2hr (OF1) in early-adolescent (p23/24; n = 24) and adult (p73-75; n = 24) male rats. Each age group's total distance traveled was ranked from highest to lowest, and divided into tertiles; the top 1/3 of the distribution was classified as High Responders (n = 8) and the bottom 1/3 of the distribution was classified as Low Responders (n = 8). Adolescent High/Low-Responder rankings were not significantly correlated over time and therefore could not be used to classify adolescents. When the early adolescent group's locomotor activity over time was compared to the adult High- and Low-Responder locomotor activity for OF1, it was clear that all adolescents resembled adult Low Responders. Values shown are group means of distance traveled over time, in 5min bins. Error bars and the activity of the middle 1/3 of the adult population (n = 8) are omitted for clarity.

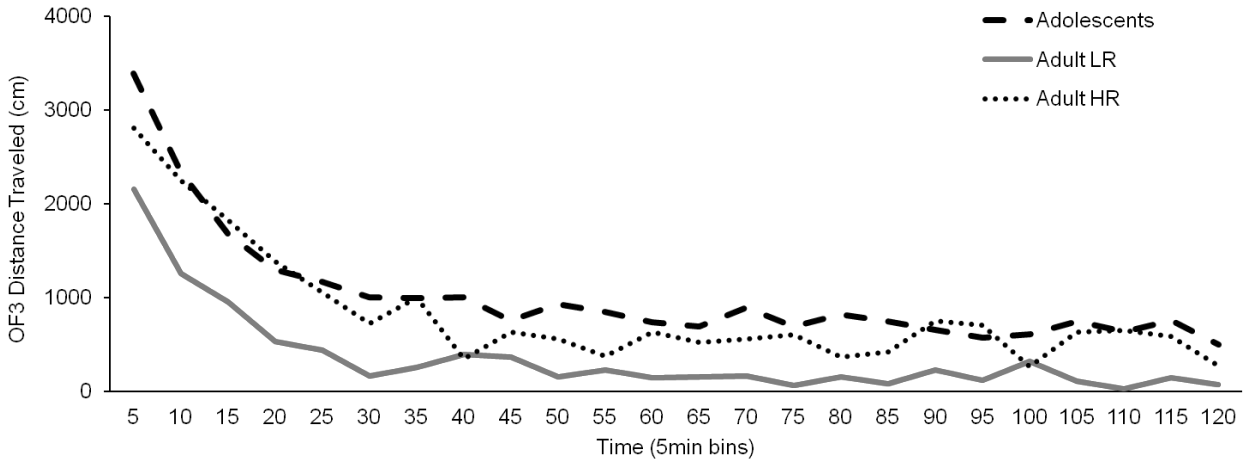


Figure A.3. Late-adolescent locomotor activity in a subsequent Open Field test resembles adult High Responder activity.

Locomotor activity in a novel environment was recorded for 2hr (OF3) in late-adolescent (p58/59; n = 24) and adult (p130-132; n = 24) male rats, 5 (adolescent) or 8 (adult) weeks after an initial Open Field test (OF1). Adults were classified as High Responders (HR; n = 8) and Low Responders (LR; n = 8) in OF1. Adolescent High/Low-Responder classifications from OF1were not significantly correlated over time and therefore were not applicable. When the late adolescent group's locomotor activity over time was compared to the adult High- and Low-Responder locomotor activity for OF3, it was clear that all adolescents resembled adult High Responders. Values shown are group means of distance traveled over time, in 5min bins. Error bars and the activity of the middle 1/3 of the adult population (n = 8) are omitted for clarity.

Table A.2. Late-adolescent rats classified as High- and Low-Responders do not differ in anxiety-like behaviors in the Elevated Plus Maze.

In a separate experiment using adolescent Controls (exposed to equivalent conditions as those discussed in **Chapter 4**), late-adolescent male rats (n = 12) were given a 2hr Open Field test on postnatal day (p) 58, followed by a 5min Elevated Plus Maze test three days later on p61. Rats' total distance traveled was divided into tertiles; the top 1/3 of the distribution was classified as high responders (HR; n = 4) and the bottom 1/3 of the distribution was classified as low responders (LR; n = 4). Total distance traveled significantly differed between High- and Low-Responders [W = 25, p=0.029]. When measures of anxiety-like behavior on the Elevated Plus Maze were compared between High- and Low-Responder groups using non-parametric analyses, no significant differences were found for: percent open arm entries (calculated as [number of open arm entries / open + closed arm entries] x 100), percent open arm time (calculated as [open arm time / 300s] x 100), or latency to first enter an open arm [all p>0.05]. OA: Open Arm of the Elevated Plus Maze. Values show group means ±SEMs. *p≤0.05 for between-group comparisons.

	LR	**HR**
OF Total distance Traveled (cm)	**9345 (1649)**	**27341 (3698)***
EPM %Open Arm Entries	21 (10)	25 (6)
EPM % OA Time	23 (9)	34 (5)
EPM Latency to 1st OA Entry (s)	112 (63)	56 (26)

APPENDIX B

APPETITIVE CUE-EVOKED ERK SIGNALING IN THE NUCLEUS ACCUMBENS REQUIRES NMDA AND D1 DOPAMINE RECEPTOR ACTIVATION AND REGULATES CREB PHOSPHORYLATION

This appendix has been published as:

Kirschmann EKZ, Mauna JC, Willis CM, Foster RL, Chipman AM, Thiels E. 2014. Appetitive cue-evoked ERK signaling in the nucleus accumbens requires NMDA and D1 dopamine receptor activation and regulates CREB phosphorylation. *Learning & Memory* [in press].

It is unchanged in content, with the exception of minor formatting alterations, and supplemental information (Tables 1-2) was added to the end of the main body of the article.

My contributions include participation in study design, data collection and analysis, and writing the manuscript under the direction of Dr. Thiels. Dr. Thiels and Jocelyn Mauna contributed to study design and manuscript preparation. All authors approved the final manuscript.

ABSTRACT

Conditioned stimuli (CS) can modulate reward-seeking behavior. This modulatory effect can be maladaptive and has been implicated in excessive reward seeking and relapse to drug addiction. We previously demonstrated that exposure to an appetitive CS causes an increase in the activation of extracellular signal-regulated kinase (ERK) and cyclic AMP response element-binding protein (CREB) in the nucleus accumbens (NAc) of rats, and that CS-evoked ERK activation is critical for CS control over reward seeking (Remus & Thiels, 2013; Shiflett et al., 2008; 2009). To elucidate the mechanism that mediates CS-driven ERK activation in the NAc, we selectively blocked NMDA glutamate or D1 dopamine receptors in the NAc. To determine whether CS-driven ERK and CREB activation are linked, we selectively blocked ERK signaling in the NAc. We found that both NMDA and D1 receptors are critical for CS-driven ERK signaling in the NAc, and that this recruitment of the ERK cascade is responsible for increased CREB activation in the presence of the CS. Our findings suggest that activation of the NMDAR-D1R/ERK/CREB signal transduction pathway plays a critical role in the control of reward-seeking behavior by reward-predictive cues.

INTRODUCTION

Environmental cues that have been paired repeatedly with reward come to predict the reward. These so-called conditioned stimuli (CSs) can have powerful control over behavior: for instance, exposure to a CS enhances an organism's seeking, or working for, the reward predicted by the CS (Cardinal et al., 2002; Estes, 1948; Everitt & Robbins, 2005; Lovibond, 1983). The potentiating effect of a CS on reward-seeking has obvious beneficial consequences by enhancing an organism's ability to procure valuable resources in its environment (Day & Carelli, 2007; Everitt et al., 1999). However, it also can be maladaptive and has been implicated in excessive

reward intake and addiction, including relapse to drug use (Corbit & Janak, 2007; Gipson et al., 2013; Grimm et al., 2002; LeBlanc et al., 2012). It therefore is important to gain understanding of the mechanisms that underlie CS modulation of reward-seeking behavior.

A brain region critical for CS control over instrumental behavior is the nucleus accumbens (NAc) (Cardinal et al., 2002; Everitt et al., 1999; Kelley et al., 2005; Zahm, 2000). This ventral striatal structure receives broad glutamatergic input from the prefrontal cortex, amygdala, subiculum, and thalamus, and dopaminergic input from the ventral tegmental area (Groenewegen et al., 1999; Zahm, 2000). In turn, it sends projections to motor areas, such as the ventral pallidum and the substantia nigra (Groenewegen et al., 1999; Mogenson et al., 1980; Zahm, 2000). We previously showed that exposure to a food-predicting CS evokes an increase in activation of extracellular signal-regulated kinase (ERK) in the NAc (Remus & Thiels, 2013; Shiflett et al., 2008). Importantly, activation of the ERK pathway is necessary for CS-modulation of reward-seeking behavior: in animals that received intra-accumbal infusions of an inhibitor of ERK activation, the CS failed to potentiate reward seeking (Shiflett et al., 2008). Here, we investigated the signaling events up- and downstream of CS-evoked ERK activation in the NAc.

Studies have identified a critical role for glutamatergic transmission, particularly through N-methyl D-aspartate receptors (NMDARs), in striatal ERK activation (Jenab et al., 2005; Mao et al., 2004; Schwarzschild et al., 1999; Valjent et al., 2000; Vincent et al., 1998). Thus, CS-induced ERK activation in the NAc may be mediated through a NMDAR-dependent mechanism. On the other hand, D1 dopamine receptors (D1Rs) also were found to be involved in striatal ERK activation (Bertran-Gonzalez et al., 2008; Borgkvist et al., 2008; Fricks-Gleason & Marshall, 2011; Valjent et al., 2004; 2005). Accordingly, both NMDARs and D1Rs may be targets for manipulating accumbal ERK signaling in the presence of a CS. The first goal of our

study therefore was to determine the role of these two receptor types in accumbal ERK activation evoked by an appetitive CS.

Our previous work showed that exposure to a food-predicting CS increases not only ERK activation but also phosphorylation of the transcriptional regulator cyclic AMP response element-binding protein (CREB) in the NAc (Shiflett et al., 2009). These two signaling events may be linked. CREB is well-known to be one of the downstream targets of the ERK signaling cascade (Xing et al., 1998) [for review see: (Carlezon Jr et al., 2005; Shaywitz & Greenberg, 1999; Thomas & Huganir, 2004)]. However, CREB phosphorylation alternatively can be mediated by other signaling pathways, including an ERK-independent protein kinase A (PKA) pathway or a calcium/calmodulin-dependent kinase IV (CaMKIV) pathway (Dash et al., 1991; Gonzalez & Montminy, 1989; Gonzalez et al., 1989; Matthews et al., 1994; Sheng et al., 1991). Therefore, the second goal of our study was to determine whether the increase in CREB phosphorylation caused by exposure to an appetitive CS is dependent on CS-evoked ERK signaling in the NAc. Our experiments show that both NMDARs and D1Rs are critical for triggering increased ERK signaling in the NAc in the presence of an appetitive CS, and that this recruitment of the ERK cascade leads to increased CREB phosphorylation in the presence of a reward-predictive cue.

MATERIALS AND METHODS

Animals

A total of 55 male rats (*Sprague Dawley*; 275-300g at arrival; Hilltop Lab Animals, Scottdale, PA) were used. Animals were housed singly under 12/12hr conditions (lights on at 7:00am). Until recovery from surgery (see below), rats had *ad libitum* access to standard rat chow (LabDiet IsoPro Rodent Chow) and tap water. Five to seven days after surgery, rats were placed

on a restricted diet of 15-20g rat chow per day to maintain their body weight at ~85% of similar-aged free-feeding rats. A schema of the experimental phases described in detail below is depicted in Figure B.1A. All procedures were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee, and were in accordance with the Guide for the Care and Use of Laboratory Animals commissioned by the National Institutes of Health.

Surgical Procedures

Within two to three days after arrival, rats were implanted bilaterally with cannulae aimed at the NAc, as described previously (Shiflett et al., 2008). Briefly, rats were anesthetized with ketamine (Ketaset, 85mg/kg intraperitoneal, i.p.; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (AnaSed, 8mg/kg i.p.; Lloyd Laboratories, Shenandoah, IA), and positioned in a stereotaxic apparatus. An incision was made to expose the skull, and two small holes were drilled (coordinates relative to Bregma: 1.3mm anterior, ± 1.7mm lateral) (Paxinos & Watson, 2007). A 26-gauge stainless steel guide cannula (6mm long; Plastics One, Roanoke, VA) was lowered into each hole 5.4mm ventral relative to dural surface, and fixed to the skull with cyanoacrylate glue and dental cement before the skin was sutured closed. Rats were given acetaminophen orally (300mg/kg/day) for two days after surgery.

Behavioral Procedures

Behavioral experiments were conducted in the Rodent Behavioral Analysis Core of the University of Pittsburgh Schools of Health Sciences. Procedures were as described previously (Remus & Thiels, 2013; Shiflett et al., 2008; 2009). Training took place in operant chambers (30cm x 23cm x 23cm; Med Associates, St. Albans, VT) equipped with a house light, a floor with metal bars, a loudspeaker that delivered a 3kHz-80db tone when activated, and a food cup attached to a pellet dispenser. When initiated, the pellet dispenser released a single 45mg dustless

food pellet (BioServe, Frenchtown, NJ) into the food cup. An infrared photo beam, emitted and detected via a source and a sensor placed immediately to the left and the right side of the food cup, was used to measure head insertions into the cup (i.e., food cup-approaches). Each operant chamber was housed in a sound-attenuating cubicle with a background noise-generating fan. Chambers were controlled by and data recorded with Med-PC software (Med Associates).

About four days after onset of food-restricted diet, rats were habituated to the conditioning chamber in a single 30min session with the house light illuminated. Beginning the next day, rats received a daily session of appetitive Pavlovian conditioning for five consecutive days. Sessions began with illumination of the house light and lasted 45-50min. A 90sec, 3kHz-80db tone served as the CS, and was presented a total of eight times with a variable inter-trial interval (ITI; tone off → tone on, mean = 4min). For rats in the experimental condition (Tone+Food group; T+F), three food pellets were delivered on a random VT20sec schedule during the last 60sec of each CS (Figure B.1B, left). For rats in the control group (Tone-only group; TO), which served to assess basal ERK activation, the delivery of food pellets was omitted (Figure B.1B, right). Testing occurred 24hr after the last Pavlovian conditioning session. The test session began with the illumination of the house light and lasted approximately 15min. A total of four tone presentations occurred at a variable ITI (mean = 4min). During training and testing, food cup-approaches were recorded during the first 30sec of the tone (when no pellets were delivered) and during 30sec preceding tone onset (i.e., during the ITI; preCS). Immediately after the test session, rats were anesthetized with chloral hydrate (300mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO; dissolved in 150mM NaCl) and either decapitated or transcardially perfused with 150mM NaCl, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB). In the case of decapitation (n = 46 rats), brains were removed rapidly, briefly immersed in isopentane (Acros

Organics, Morris Plains, NJ) on dry ice, and stored at -80^{o}C until Western blot analysis (see below). In the case of transcardial perfusion (n = 9 rats), brains were removed, placed in a 20% sucrose solution (dissolved in PB) until they sank, frozen, sectioned at a thickness of 40µm on a cryostat at -16°C, and stored in cryopreservative at -20°C until immunohistochemical staining (see below).

Intra-NAc Microinfusions

To investigate the role of specific receptors in CS-evoked ERK activation and the role of ERK in CS-evoked CREB phosphorylation, rats received intra-NAc infusions of specific reagents shortly before testing as described below (see Figure B.1C for representative cannula placements). Unilateral drug infusions were combined with unilateral vehicle infusions in the same animal, in order to allow for intra-individual comparisons of NAc tissue (drug vs. vehicle). To familiarize rats with the infusion procedure, rats received a mock infusion two to three days prior to the test day. Wire stylets were removed from the guide cannulae, short infusion cannulae (33-gauge, projecting 0.5mm past the guide, Plastics One; attached via PVC tubing to a Hamilton syringe [Reno, NV]) were inserted, and 0.5µl of 150mM NaCl solution was infused bilaterally at a rate of 0.5µl/min. Infusion cannulae were left in place for an additional minute. Microinfusions on the test day were conducted in the same manner, except that (1) the infusion cannulae extended 2.2mm past the tip of the guide and into the NAc, and (2) the infusates were as follows. To examine the contribution of NMDARs, rats (n = 9 T+F, 10 TO) received immediately before testing the NMDAR antagonist D-(2R)-amino-5-phosphonovaleric acid (APV; Tocris Bioscience, Ellisville, MO; 2mg/ml dissolved in 150mM NaCl; final dose: 1µg) in one NAc and an equal volume (0.5µL) of vehicle in the other. To examine the contribution of D1-type dopamine receptors, rats (n = 6 T+F, 6 TO) received immediately before testing the D1R

antagonist R(+)-SCH-23390 (SCH; Sigma-Aldrich; 0.6mg/ml dissolved in 150mM NaCl; final dose 0. 3µg) in one NAc and an equal volume (0.5µL) of vehicle in the other. To determine whether CS-evoked increases in ERK and CREB phosphorylation are linked, rats (n = 8 T+F, 7 TO) received 30min before testing the specific mitogen-activated protein kinase kinase/ERK inhibitor U0126 (Millipore, Burlington, MA; 2mg/ml dissolved in 50% dimethyl sulphoxide [DMSO; Sigma-Aldrich] / 50% 150mM NaCl [50% DMSO]; final dose: 1µg) in one NAc and an equal volume (0.5µL) of vehicle in the other. The side receiving drug was counterbalanced across subjects. Correct cannulae placements were verified visually when excising tissue samples for Western blot analysis or with immunohistochemical staining (see below). Examples are depicted in Figure B.1C.

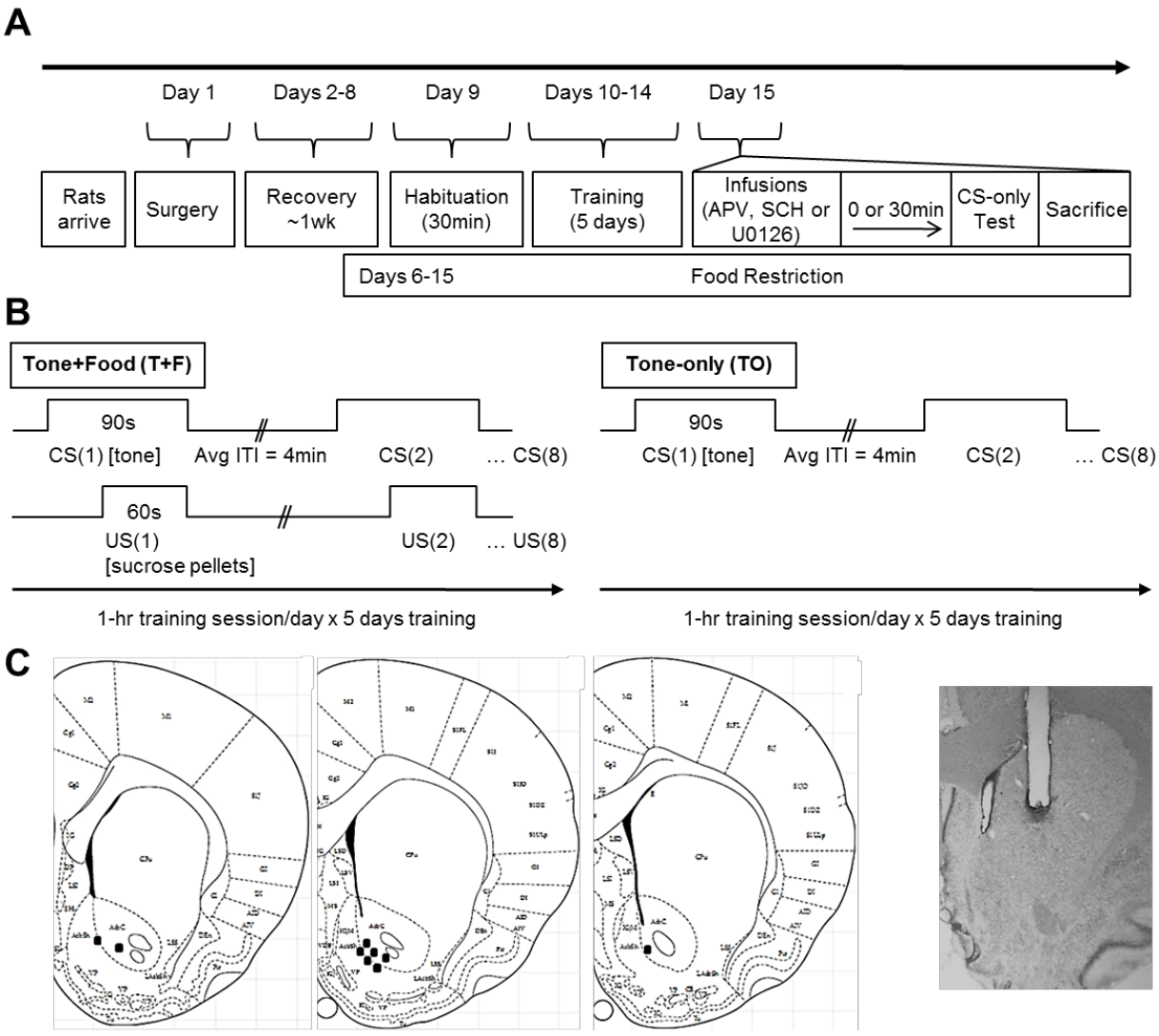


Figure B.1. Experimental Details.

A) Experimental Timeline. After 1 day of habituation and 5 days of training, rats were tested under one of 3 drug conditions: 1) immediately after infusion of APV/vehicle; 2) immediately after infusion of SCH/vehicle; or 3) 30min after infusion of U0126/vehicle. B) Trial timelines for the group that received paired Tone+Food presentations (T+F group) and the group that received Tone-only presentations (TO control group) during training. C) Cannulae placements for immunohistochemical experiments. Placements spanned from +1.0 to +1.7mm relative to Bregma. Representative track end points are indicated by circles on plate images shown on the left (modified from Paxinos & Watson, 2007, with permission from Elsevier) and a Nissl stain image of a representative guide cannula placement is shown on the right. Comparable placements were observed for Western blot experiments.

Western Blot Analysis

As described previously (Remus & Thiels, 2013; Shiflett et al., 2008; 2009), NAc tissue samples were excised from three consecutive 400µm-thick sections using a tissue punch (diameter = 2mm; Fine Science Tools, Foster City, CA) placed over the NAc so as to include about equal parts of the NAc core and shell. Tissue samples were homogenized in a buffer containing 150mM NaCl, 1mM EDTA, 50mM Tris (pH 7.4), 0.05% SDS, 1%Triton-X-100, 1mM dithiothreitol (DTT), 2mM sodium fluoride (NaF), 1mM orthovanadate, 2mM sodium pyrophosphate, 100x protease inhibitor cocktail, and 1mg/ml pepstatin (Sigma-Aldrich; Millipore; BioRad, Hercules, CA; Fisher Scientific, Pittsburgh, PA). Homogenate was centrifuged for 15min at 14,000rpm, the supernatant was collected, and protein concentration was determined in triplicates using a bicinchoninic acid assay (Pierce BCA Protein Assay, Fisher Scientific and 4% Copper(II) sulfate solution, Sigma-Aldrich). Samples were diluted to a uniform protein concentration with homogenization buffer and sample buffer containing 2.5M Tris (pH 6.8), 40% glycerol, 8% SDS, 0.05% bromophenol blue, and 30mg/ml DTT (Sigma-Aldrich, BioRad, Fisher Scientific), and heated to 95°C for 5min. Fifty µg of protein/sample were loaded for resolution by SDS-PAGE and electrophoretically transferred to Immobilon membranes (Fisher Scientific). For pERK analyses, membranes were incubated for 1hr at room temperature in a Tris-buffered saline (0.05M Tris [pH 7.9], 0.15M NaCl) plus 0.1% Tween 20 (Sigma-Aldrich) solution (TBST) containing 5% dried nonfat milk, and then incubated overnight with an antibody that specifically recognizes T-202/183- and Y-204/185-phosphorylated, i.e., active p44/42 MAPK (pERK, 1:2500 dilution in 5% bovine serum albumin [BSA; dissolved in TBST]). Antigen binding was visualized with a HRP-linked secondary antibody (anti-rabbit, 1:5000 dilution in 5% milk) and enhanced chemiluminescence reagent (Lumiglo; Cell Signaling,

Beverly, MA). Blot images were captured with a CCD camera (Hamamatsu Photonics, Japan) and analyzed using densitometry software (UVP Labworks, Upland, CA). Membranes were stripped by incubation at 50°C for 45min in a solution containing 62.5mM Tris (pH 6.7), 2% SDS, and 0.62% β-mercaptoethanol, blocked in 5% milk, and reprobed with an antibody that specifically recognizes phosphorylated and unphosphorylated, i.e., total p44/42 MAPK (tERK, 1:2500 dilution in 5% BSA). Antigen binding (secondary antibody: anti-rabbit, 1:5000 dilution in 5% milk) was visualized and blot images captured as described above. For pCREB analyses, membranes were cut horizontally at 50kD, and both halves were incubated for 1hr at room temperature in TBST containing 5% milk. The top half of each membrane was incubated overnight with an antibody that specifically recognizes neuN (protein loading control; 1:5000 dilution in 5% BSA), and the bottom half with an antibody that specifically recognizes S-133 phosphorylated CREB (pCREB, 1:1000 dilution in 5% BSA). Antigen binding was visualized (secondary antibody for neuN: anti-mouse, 1:5000 dilution in 5% milk; for pCREB: anti-rabbit, 1:5000 dilution in 5% milk) and blot images captured as described above. Antibodies were obtained from Cell Signaling (pERK, tERK, anti-rabbit, and anti-mouse) and Millipore (pCREB and neuN).

Immunohistochemistry

Nine additional T+F rats were trained, infused, and tested as described above, to conduct immunohistochemical analyses. Three rats were infused with vehicle/APV, three rats with vehicle/SCH, and three rats with vehicle/U0126. We probed coronal tissue sections for pERK1/2 or pCREB immunoreactivity as described previously (Remus & Thiels, 2013; Shiflett et al., 2008; 2009). Briefly, free-floating sections were brought to room temperature (RT) over 30min, washed with sequential rinses in 50mM Tris Buffered Saline (TBS; pH 7.6) on a rocker table,

pretreated with an antigen retrieval Tris-Tween buffer at 80°C for 20min, rinsed again several times in 50mM TBS, and then bathed in 50mM TBS containing 0.4% Triton X-100 and 10% normal goat serum at RT for 1hr. Consecutive serial sections were incubated with an antibody that selectively recognizes either T-202/183- and Y-204/185-phosphorylated ERK1/2 (pERK, 1:1000 dilution; Cell Signaling) or S-133 phosphorylated CREB (pCREB, 1:1000 dilution; Millipore) in 50mM TBS containing 0.4% Triton X-100 and 10% normal goat serum at 4°C for 48-72hr. All perfusion solutions, rinses, and incubation solutions before and during incubation with the primary antibody contained 1mM NaF and 1mM orthovanadate, to prevent dephosphorylation of antigen. After washing in 50mM TBS over 45min, sections were incubated in biotinylated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) in 50mM TBS containing 0.4% Triton X-100 for 90min, followed by incubation in avidin-biotin conjugate (ABC; 1:500 for each A and B reagent; Vector Laboratories, Burlingame, CA) in 50mM TBS containing 0.4% Triton X-100 for 90min at RT on a rotator. After washing sections in 50mM TBS, immunostaining was visualized with a substrate solution containing diaminobenzidine 0.6mg/ml, 0.03% H_2O_2, and 4mM $NiCl_2$ in TBS. Sections were washed several times and then mounted on gelatin-coated slides, dried at RT, dehydrated in ethanol, cleared in xylene, and coverslipped with Cytoseal 60 (Fisher Scientific). Images of sections stained for pERK1/2 or pCREB were captured using a 10X objective and a digital camera (Micrometrics 3.2 MP) mounted on a light microscope (Leitz Orthoplan 2). The number of pERK- immunopositive cells or density of pCREB-immunopositive nuclei in the NAc were estimated from digital images using NIH ImageJ software.

Statistical Analyses

For Pavlovian approach behavior, number of photo beam breaks during the CS and preCS intervals was summed for each session. A difference score was calculated for each animal by subtracting CS from preCS beam-break rates for that session. Difference scores, a measure of discriminated approach, were compared using analyses of variance (ANOVAs) with group as between-subject factor and training day as within-subject factor, followed by post-hoc pairwise comparisons using t tests with Bonferroni's correction. For Western blot analysis, ratios of pERK immunoreactivity to tERK immunoreactivity or pCREB immunoreactivity to neuN immunoreactivity were calculated for each sample and then normalized to a loading control included on each membrane. Comparisons were made using ANOVAs with group as between-subject factor and drug condition as within-subject factor, and followed by post-hoc t tests with Bonferroni's correction (Remus & Thiels, 2013; Shiflett et al., 2008; 2009). Statistical analyses were performed using the SPSS software package version 19.0 (SPSS, Chicago, IL). Significance level was set to $\alpha \leq 0.05$.

RESULTS

Conditioned Cue-Evoked Increases in ERK Signaling in the NAc Are Mediated by NMDA and D1 Receptor Activation

To determine the receptor pharmacology of the CS-evoked increase in ERK activation in the NAc we observed previously (Remus & Thiels, 2013; Shiflett et al., 2008), we infused the specific NMDAR antagonist D-(2R)-amino-5-phosphonovaleric acid (APV; 1µg in 0.5µL) into the NAc on one side and vehicle solution (150mM NaCl; 0.5µL) into the NAc on the other side shortly before the tone test used for examination of CS-evoked ERK activation. Discriminative food cup approach behavior (rate of food cup approaches during the first 30sec of the tone minus

rate of food cup approaches during 30sec preCS) of the Tone+Food (T+F) group and the Tone-only (TO) control group during training is shown in Figure B.2A. Whereas the two groups did not differ from one another at the beginning of training, only rats in the T+F group (n = 9) but not rats in the TO group (n = 10) developed preferential food cup approaches during the CS over the course of training [group X day interaction: $F(4, 68) = 5.38$, $p=0.001$]. Discriminative food cup approach of the two groups on the test day, shortly after intra-NAc drug infusion, is depicted in Figure B.2B. As was the case during the later phases of training, the discrimination rates of rats in the T+F group were significantly higher than those by rats in the TO group [$t(17) = 5.65$, $p<0.001$, two-tailed]. These results indicate that unilateral NMDAR blockade in the NAc does not interfere with discriminative responding to the CS.

The effect of unilateral NMDAR blockade on NAc ERK2 activation evoked by exposure to the tone-CS is shown in Figure B.2C. We examined ERK2 (p42 isoform) because the activation state of specifically this isoform was shown to be altered during learning and reward-motivated behavior (Ferguson et al., 2006; Girault et al., 2007; Mazzucchelli et al., 2002; Shiflett et al., 2008). Focusing first on the vehicle-infused side of the NAc, we found that pERK2 immunoreactivity relative to tERK2 immunoreactivity, i.e., ERK2 activation, was about two-fold higher in the NAc of rats in the T+F group than in the NAc of rats in the TO group. This finding is in agreement with previous observations of increased ERK2 activation in the NAc upon exposure to an appetitive CS (Remus & Thiels, 2013; Shiflett et al., 2008). Importantly, this increase in ERK2 activation in rats of the T+F group was markedly reduced in the presence of APV; in contrast, the NMDAR antagonist had no effect on ERK2 activation in the NAc of the TO control group [group X drug interaction: $F(1,17) = 6.19$, $p=0.024$]. Post-hoc comparisons showed that the ERK2 signal was significantly higher in the vehicle- compared to APV-infused

side only in rats of the T+F group ($p=0.004$). Figure B.2D depicts a representative immunohistochemical image of pERK-immunoreactive cells in the vehicle-infused NAc (top) and the APV-infused NAc (bottom) of a T+F rat, and Supplemental Table B.1 shows quantification of pERK-staining examined in 3 T+F rats. The density of pERK-immunoreactive (pERK+) cells on the vehicle-infused side was similar to levels we observed previously in T+F trained rats whereas that on the APV-infused side was similar to levels we observed previously in TO controls (Remus & Thiels, 2013). Furthermore, similar to the subregional patterns we described in our earlier work comparing T+F trained rats and TO controls, the number of pERK+ cells was higher in the shell subregion of the NAc than in the core subregion, but the proportional difference between the vehicle- and the APV-infused side was comparable across subregions. Taken together, these findings show that NMDAR activation critically contributes to CS-evoked increases in ERK2 signaling but does not play a role in the regulation of basal ERK2 signaling.

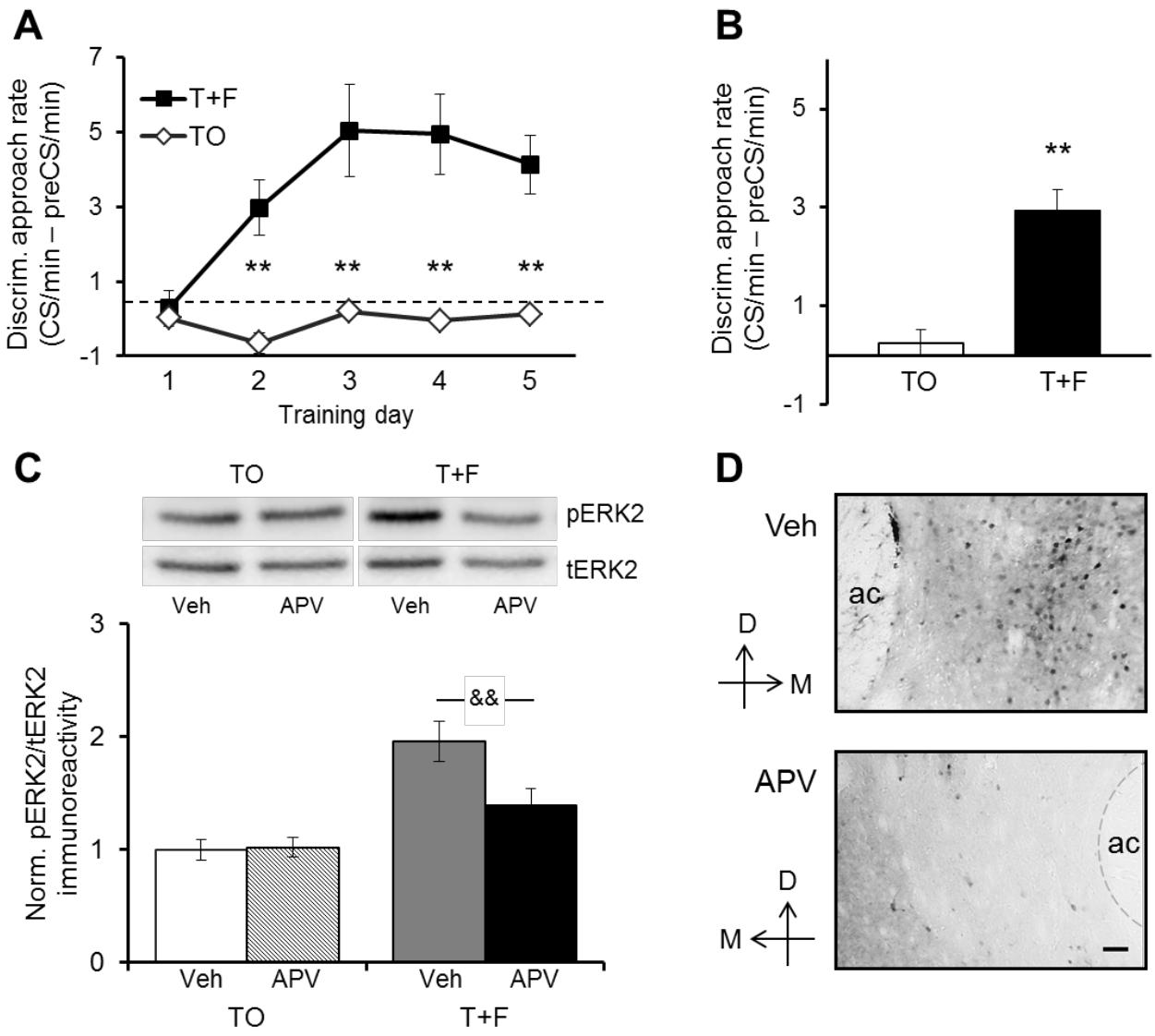


Figure B.2. NMDA receptor blockade in the NAc disrupts conditioned cue-evoked ERK activation.

A) Discriminative food cup approach during daily training sessions for rats in the Tone+Food group (T+F; n = 9) and the Tone-only control group (TO; n = 10). T+F rats preferentially approached the food cup during the tone (CS) compared to the preCS period, whereas TO controls did not exhibit discriminative approach behavior. Values show group means ± SEMs. **p<0.01 for between-group comparisons. B) Discriminative approach behavior during the CS-only test, immediately after infusion of APV (1µg/0.5µl) into one NAc and vehicle solution (150mM NaCl, 0.5µl) into the other NAc. Unilateral intra-NAc NMDA receptor blockade had no effect on discriminative approach behavior by the T+F group. Values show group means ± SEMs. **p<0.01. C) ERK2 activation (pERK2

immunoreactivity relative to tERK2 immunoreactivity in the same sample) in NAc samples harvested from rats of the T+F group and the TO control group shortly after the CS-only test. Ratios of pERK/tERK immunoreactivity are expressed relative to the same ratio observed in vehicle-infused NAc samples from TO control rats included on the same membrane. Values show group means ± SEMs. &&p<0.01 for within-group comparisons. Representative Western blots of the vehicle- and the APV-infused sides of a rat of the T+F group and a rat of the TO group are shown above. D) Representative immunohistochemical image of pERK-immunoreactive cells in the NAc of a rat from the T+F group. Top panel: NAc side infused with vehicle solution; bottom panel: NAc side infused with APV. Similar differences in staining between the vehicle- and APV-infused sides were observed in other T+F rats (n = 3). Scale bar, 50μm. D: Dorsal; M: Medial; ac: anterior commissure. To help discern the border of the ac, a thin grey dashed lined was added in some of the images.

To determine whether the CS-evoked increases in ERK signaling also require D1Rs, we repeated essentially the same experiment as described above with a new set of animals, except that we infused the specific D1-like receptor antagonist SCH23390 (SCH; 0.3µg in 0.5µL) into the NAc on one side and vehicle solution (150mM NaCl; 0.5µL) into the NAc on the other side shortly before the tone test. Discriminative food cup approach behavior of the two groups during training is shown in Figure B.3A. Similar to our previous observations, discriminative food cup approach behavior developed only in rats of the T+F group (n = 6) but not in rats of the TO group (n = 6) [group X day interaction: $F(4, 40) = 3.48$, $p=0.016$]. On test day, immediately after unilateral infusion of SCH, the group differences were maintained (Figure B.3B). Discriminative food cup approach by rats in the T+F group was significantly higher than that of rats in the TO group [$t(10) = 5.15$, $p<0.001$]. Thus, unilateral D1R blockade leaves discriminative responding to the CS intact.

The effect of unilateral D1R blockade on NAc ERK2 activation evoked by exposure to the tone-CS is shown in Figure B.3C. Once again, ERK2 activation on the vehicle-infused side of the NAc was about two-fold higher in rats of the T+F group than rats of the TO group. This increase in ERK2 activation in the NAc of rats in the T+F group was reduced in the presence of SCH, whereas the D1R antagonist had no effect on ERK2 activation in the NAc of TO rats [group X drug interaction: $F(1,10) = 10.73$, $p=0.008$]. Post-hoc tests confirmed that ERK2 activation was significantly higher in the vehicle- compared to the SCH-infused NAc of rats from the T+F group ($p=0.003$), but did not differ between drug conditions in rats from the TO group. Figure B.3D depicts a representative immunohistochemical image of pERK-immunoreactive cells in the vehicle-infused NAc (top) and the SCH-infused NAc (bottom) of a T+F rat, and Supplemental Table B.1 shows quantification of pERK+ cells in vehicle-infused

NAc versus SCH-infused NAc of 3 T+F rats. The differential pattern of density of pERK+ cells between the vehicle-infused and the drug-infused side and between the NAc core and the NAc shell was similar to the pattern we found after unilateral APV infusion (see above) and observed previously in T+F trained and TO control rats, respectively (Remus & Thiels, 2013). The findings with the D1R antagonist show that basal ERK2 activation is not regulated in a D1R-dependent fashion but that D1R activation is necessary for increased ERK2 signaling after exposure to an appetitive CS. Taken together, our results indicate that both NMDARs and D1Rs are critical for the regulation of NAc ERK signaling by reward-predictive cues.

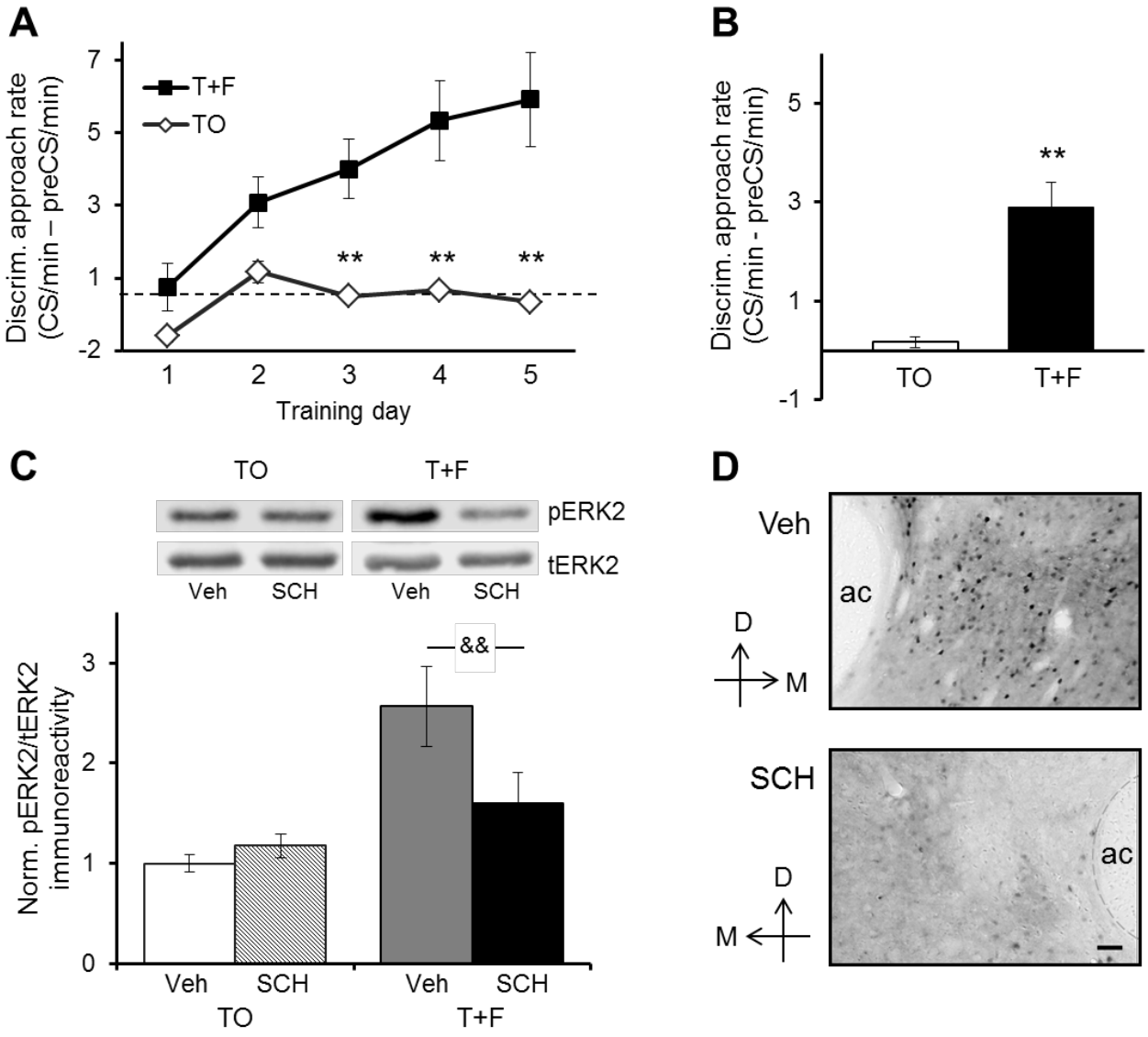


Figure B.3. D1 receptor blockade in the NAc disrupts conditioned cue-evoked ERK activation.

A) Discriminative food cup approach during daily training sessions for rats in the Tone+Food group (T+F; n = 6) and the Tone-only control group (TO; n = 6). T+F rats preferentially approached the food cup during the CS compared to the preCS period, whereas TO controls did not exhibit discriminative approach behavior. Values show group means ± SEMs. **p<0.01 for between-group comparison. B) Discriminative approach behavior during the CS-only test, immediately after infusion of SCH (0.3µg/0.5µl) into one NAc and vehicle solution (150mM NaCl, 0.5µl) into the other NAc. Unilateral intra-NAc D1 receptor blockade had no effect on discriminative approach behavior by the T+F group. Values show group means ± SEMs. **p<0.01. C) ERK2 activation (pERK2

immunoreactivity relative to tERK2 immunoreactivity in the same sample) in NAc samples harvested from rats of the T+F group and the TO control group shortly after the CS-only test. Ratios of pERK/tERK immunoreactivity are expressed relative to the same ratio observed in vehicle-infused NAc samples from TO control rats included on the same membrane. Values show group means ± SEMs. &&p<0.01 for within-group comparisons. Representative Western blots of the vehicle- and SCH-infused sides of a rat of the T+F group and a rat of the TO group are shown above. D) Representative immunohistochemical image of pERK-immunoreactive cells in the NAc of a rat from the T+F group. Top panel: NAc side infused with vehicle solution; bottom panel: NAc side infused with SCH. Similar differences in staining between the vehicle- and SCH-infused sides were observed in other T+F rats (n = 3). Scale bar, 50μm. D: Dorsal; M: Medial; ac: anterior commissure. To help discern the border of the ac, a thin grey dashed lined was added in some of the images.

Conditioned Cue Effects on ERK Signaling Are Responsible For Cue-Evoked Increases in CREB Phosphorylation in the NAc

A major downstream target of activated ERK is the plasticity-relevant transcription factor, CREB. We previously found that exposure to a reward-predictive cue increases not only ERK activation but also phosphorylation at the transcriptionally relevant Ser-133 site of CREB in the NAc (Shiflett et al., 2009). As a first step to determine whether these two signaling events are linked, we probed NAc samples of the animals that received APV infusions immediately before the tone test (see Figure B.2) for Ser-133-phosphorylated CREB. We reasoned that if increased phosphorylation of CREB upon exposure to a reward-predictive cue lies downstream of the cue-evoked increase in ERK activation, then disruption of the CS effect on ERK should also interfere with the CS effect on CREB. Figure B.4A shows that in the presence of vehicle solution, pCREB immunoreactivity relative to neuN immunoreactivity measured in the same samples (i.e., phosphorylated CREB) is markedly higher in the NAc of T+F rats than the NAc of TO rats, similar to the CS-evoked increase in CREB phosphorylation we reported previously (Shiflett et al., 2009). In the presence of APV, the CS-evoked increase in phosphorylated CREB was greatly reduced, whereas basal levels of phosphorylated CREB were unaffected [group X drug interaction: $F(1,16) = 11.31$, $p=0.004$]. Post-hoc comparisons confirmed a significant difference between the drug- and vehicle-infused sides in the T+F group ($p=0.002$) but not the TO group.

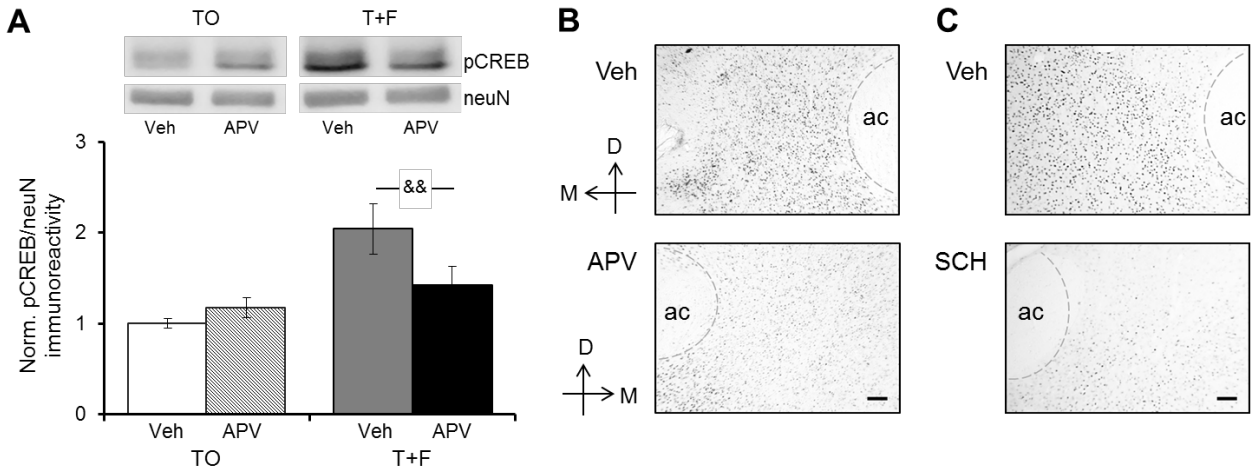


Figure B.4. NMDA or D1 receptor blockade in the NAc interferes with conditioned cue-evoked CREB phosphorylation. A) Levels of phosphorylated CREB (pCREB immunoreactivity relative to neuN immunoreactivity in the same sample) were quantified from the same NAc samples harvested from 9 T+F and 9 TO rats receiving APV/vehicle infusions and quantified for pERK (shown in Figure B.2). Ratios of pCREB/neuN immunoreactivity are expressed relative to the same ratio observed in vehicle-infused NAc samples from TO control rats included on the same membrane. Values show group means ± SEMs. &&p<0.01 for within-group comparisons. Representative Western blots of the vehicle- and APV-infused side of a T+F rat and a TO rat are shown above. B) Representative immunohistochemical image of pCREB-immunoreactive cells in the NAc of a T+F rat infused with APV/vehicle on test day. Top panel: NAc side infused with vehicle solution; bottom panel: NAc side infused with APV. C) Representative immunohistochemical image of pCREB-immunoreactive cells in the NAc of a T+F rat infused with SCH/vehicle on test day. Top panel: NAc side infused with vehicle solution; bottom panel: NAc side infused with SCH. Scale bar, 50µm. D: Dorsal; M: Medial; ac: anterior commissure. To help discern the border of the ac, a thin grey dashed lined was added in some of the images.

Figure B.4B shows representative immunohistochemical images of pCREB-immunoreactive cells in the vehicle-infused NAc (top) and the drug-infused NAc (bottom) of a T+F rat infused with the NMDAR antagonist APV. Whereas the density of pCREB staining on the vehicle-infused side is similar to what we observed previously in the NAc of T+F rats, the distinctly lighter staining pattern on the APV-infused side resembles pCREB staining levels we observed in the NAc of TO rats (Shiflett et al., 2009). Figure B.4C depicts similar immunohistochemical images of pCREB-immunoreactive cells from a representative T+F rat infused with vehicle into the NAc on one side (top) and the D1R antagonist SCH into the NAc on the other side (bottom). Again, the density of pCREB immunostaining is reduced on the drug-infused compared to the vehicle-infused side and values are similar to those we previously observed. Overall, more immunopositive cells were detected with the pCREB than the pERK antibody (Figures B.2 and B.3), most likely because the pCREB antibody is known to recognize ATF1 and CREM as well (Mattson et al., 2005). Supplemental Table B.2 shows quantification of pCREB density examined in 3 T+F rats treated with APV, and 3 T+F rats treated with SCH. In both APV and SCH immunohistochemical analyses, the proportional difference of pCREB density between the vehicle- and drug-infused NAc was greater in the core than in the shell, similar to the subregional difference in pCREB staining between TO controls and T+F rats we observed previously as well (Shiflett et al., 2009). Taken together, these results parallel the respective effects of the receptor blockers on ERK activation, and suggest that an effector of CS-evoked ERK signaling is CREB.

To test this idea directly, we trained rats as described above, except that animals received an infusion of the specific mitogen-activated protein kinase kinase/ERK inhibitor U0126 (1µg in 0.5µL) into the NAc on one side and vehicle solution (50% DMSO in 150mM NaCl; 0.5µL) into

the NAc on the other side 30min prior to the tone test for assessment of CS-driven CREB phosphorylation. Consistent with our previous findings, discriminative food cup approach behavior developed in rats of the T+F group (n = 8) but not in rats of the TO group (n = 7) [group X day interaction: $F(4, 52) = 5.72$, $p=0.001$] (data not shown). Discriminative food cup approach behavior of the two groups on the test day, after infusion of U0126 into the NAc on one side and vehicle solution into the NAc on the other side, is depicted in Figure B.5A. As was the case during the later phases of training, the discrimination rates of rats in the T+F group were significantly higher than those by rats in the TO group [$t(13) = 2.92$, $p=0.014$, two-tailed]. These results indicate that unilateral inhibition of ERK activation in the NAc does not interfere with discriminative responding to the CS.

Figure B.5B shows the effect of the MEK/ERK inhibitor on ERK activation. Whereas in the presence of vehicle solution ERK2 activation in the NAc of rats from the T+F group was markedly elevated, consistent with our previous observations [(Shiflett et al., 2008); above], this effect was completely abolished in the presence of U0126. At the same time, the MEK/ERK inhibitor had no effect on basal ERK2 activation [group x drug interaction: $F(1,13) = 10.85$, $p=0.006$]. Post-hoc comparisons confirmed that ERK2 activation was significantly higher in the vehicle- compared to U0126-infused NAc only for the T+F group ($p<0.001$) but not the TO control group. Thus, our manipulation interfered selectively with CS-evoked ERK2 activation.

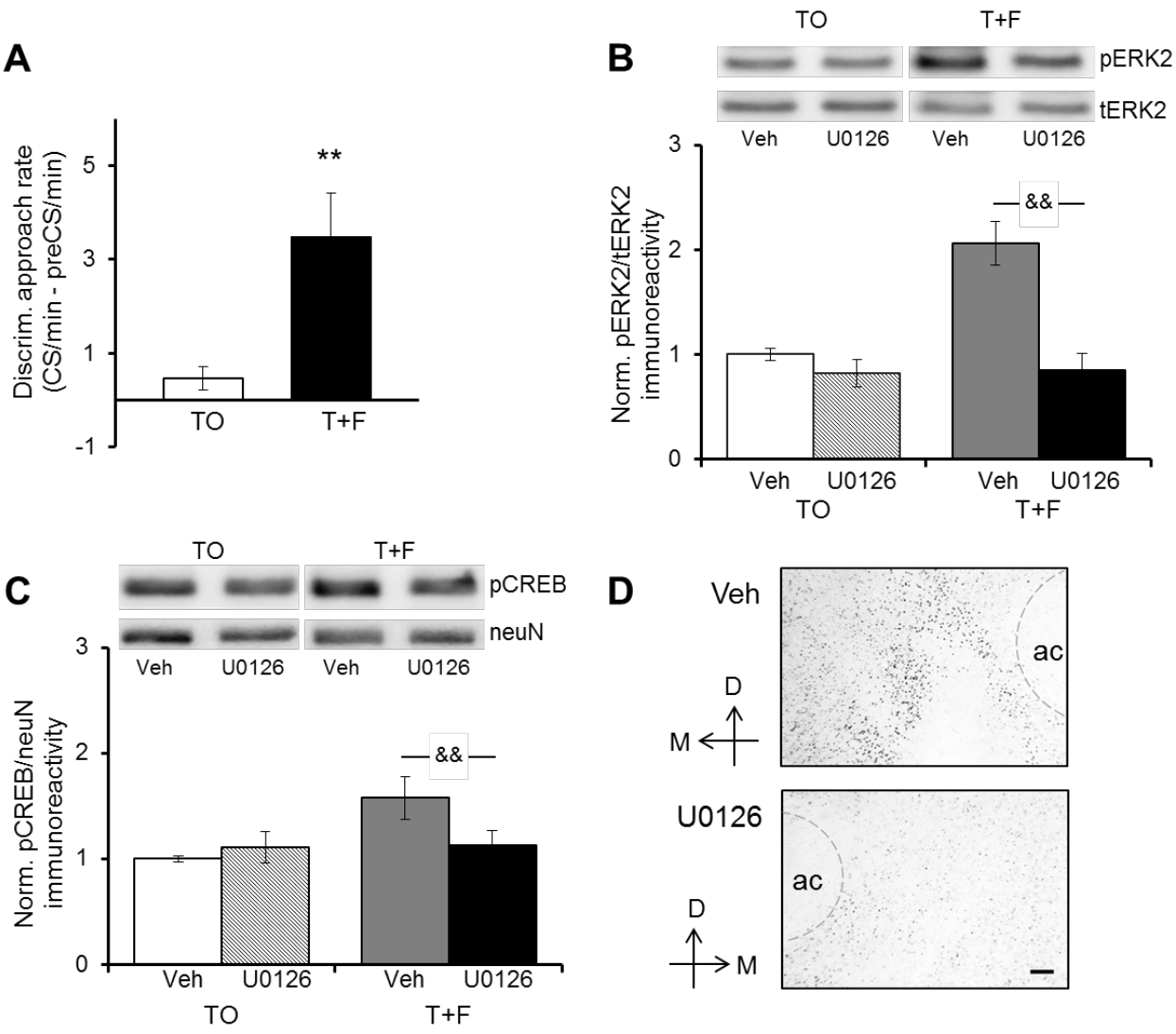


Figure B.5. Blockade of ERK activation in the NAc prevents conditioned cue-evoked CREB phosphorylation. A) Discriminative approach behavior in T+F (n = 8) and TO (n = 7) rats during the CS-only test, 30min after infusion of U0126 (1µg/0.5µl) into one NAc and vehicle solution (50% DMSO, 0.5µl) into the other NAc. Unilateral intra-NAc blockade of ERK activation had no effect on discriminative approach behavior by the T+F group. Values show group means ± SEMs. **p<0.01 for between-group comparisons. B) ERK2 activation (pERK2 immunoreactivity relative to tERK2 immunoreactivity in the same sample) in NAc samples harvested shortly after the CS-only test from rats of the T+F group and the TO control group shown in panel A. Ratios of pERK/tERK immunoreactivity are expressed relative to the same ratio observed in vehicle-infused NAc samples from TO control rats included on the same membrane. Values show group means ± SEMs. &&p<0.01 for within-group comparisons.

Representative Western blots of the vehicle- and U0126-infused sides of a rat of the T+F group and a rat of the TO group are shown above. C) CREB phosphorylation (pCREB immunoreactivity relative to neuN immunoreactivity in the same sample) from the same rats and NAc samples depicted in panels A and B, respectively. Ratios of pCREB/neuN immunoreactivity are expressed relative to the same ratio observed in vehicle-infused NAc samples from TO control rats included on the same membrane. Values show group means ± SEMs. &&p<0.01 for within-group comparisons. Representative Western blots of the vehicle- and U0126-infused sides of a rat of the T+F group and a rat of the TO group are shown above. D) Representative immunohistochemical image of pCREB-immunoreactive cells in the NAc of a T+F rat. Top panel: NAc side infused with vehicle solution; bottom panel: NAc side infused with U0126. Similar differences in staining between the vehicle- and U0126-infused sides were observed in other T+F rats (n = 3). Scale bar, 50μm. D: Dorsal; M: Medial; ac: anterior commissure. To help discern the border of the ac, a thin grey dashed lined was added in some of the images.

The effect of ERK inhibition on the level of phosphorylated CREB in the same rats is displayed in Figure B.5C. The increase in pCREB level we observed in the vehicle-infused NAc of rats in the T+F group was not observed in the U0126-infused NAc. Basal pCREB levels, on the other hand, were unaffected by the presence of the MEK/ERK inhibitor [group x drug interaction: $F(1, 13) = 10.39$, $p=0.007$]. Post-hoc comparisons confirmed that levels of phosphorylated CREB were significantly higher in the vehicle- compared to the U0126-infused NAc among rats of the T+F group ($p=0.002$) but did not differ between drug conditions among rats of the TO control group. Figure B.5D depicts a representative immunohistochemical image of pCREB-immunoreactive cells in the vehicle-infused NAc (top) and the U0126-infused NAc (bottom) of a T+F rat, and Supplemental Table B.2 shows quantification of pCREB density examined in 3 T+F rats. Similar to our findings with APV and SCH, the density of pCREB immunostaining was reduced on the drug-infused compared to the vehicle-infused side. Taken together, our results indicate that the increase in phosphorylated CREB in the NAc upon exposure to a reward-predictive cue requires ERK signaling. Furthermore, they strongly suggest a direct link between cue-evoked ERK activation and cue-evoked CREB phosphorylation in the NAc.

DISCUSSION

We previously showed that exposure to an appetitive cue causes an increase in ERK signaling and CREB phosphorylation in the NAc (Remus & Thiels, 2013; Shiflett et al., 2008; 2009), and that recruitment of the ERK pathway in the NAc is necessary for CS-modulation of reward-seeking behavior (Shiflett et al., 2008). Furthermore, we showed that the CS-driven increase in ERK activation is specific to the ventral striatum and does not occur in the dorsal striatum (Remus & Thiels, 2013; Shiflett et al., 2008). The goal of the present experiments was to identify

the synaptic signal mediating CS-evoked ERK activation in the NAc, and to establish whether CS-evoked ERK signaling and CREB phosphorylation (Shiflett et al., 2009) are linked. We found that both NMDARs and D1Rs are critical for driving ERK activation by an appetitive CS, and that the NMDAR-D1R/ERK signal transduction pathway is responsible for increased accumbal CREB phosphorylation in the presence of an appetitive CS.

NMDARs have been linked with ERK activation in the NAc [reviewed in: (Girault et al., 2007)], and are a reasonable synaptic candidate involved in CS-evoked ERK activation. First, exposure to cues signaling availability of cocaine was found to increase, and cues signaling unavailability to decrease, extracellular glutamate in the NAc (Suto et al., 2013). Second, stimulation of glutamatergic afferents in striatal slices (Sgambato et al., 1998a; 1998b; Vanhoutte et al., 1999) or bath-application of glutamate or NMDA to striatal neurons in culture (Fuller et al., 2001; Mao et al., 2004; Perkinton et al., 2002; Schwarzschild et al., 1999; Vincent et al., 1998) caused increases in striatal ERK activation. Third, NMDAR antagonists were found to interfere with glutamate-, D1R-, or drug-evoked ERK activation in the striatum (Fasano et al., 2009; Haberny & Carr, 2005; Jenab et al., 2005; Jiao et al., 2007; Mazzucchelli et al., 2002; Pascoli et al., 2011; Valjent et al., 2000; Vincent et al., 1998). Our data showing that NMDAR blockade interferes with CS-evoked ERK activation in the NAc extend these findings by demonstrating a role for NMDAR activation in accumbal ERK signaling by behaviorally relevant cues as well. Our studies do not rule out a possible enabling role of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptors (AMPARs) in the observed ERK signal. However, selective AMPAR activation causes no or only comparatively small increases in striatal ERK signaling (Fuller et al., 2001; Mao et al., 2004). Our findings, combined with the aforementioned

body of work, therefore lead us to conclude that NMDARs are the primary glutamatergic mechanism responsible for the regulation of accumbal ERK signaling by appetitive CSs.

D1Rs also have positive coupling with ERK activation in the NAc [reviewed in: (Girault et al., 2007)], and are another reasonable synaptic candidate involved in CS-driven ERK signaling. First, exposure to appetitive CSs was shown to increase extracellular dopamine in the NAc (Bassareo & Di Chiara, 1999; Cheng et al., 2003; Day et al., 2007; Phillips et al., 2003; Roitman, 2004). Second, ERK activation evoked by exposure to drug-paired contextual cues was shown to be prevented by D1R blockade and found to occur in D1R- but not D2 receptor (D2R)-expressing medium spiny neurons (MSNs) of the NAc (Borgkvist et al., 2008; Fricks-Gleason & Marshall, 2011). Stimulation of D2Rs, on the other hand, tends to have no effect or decreases ERK activation (Gerfen et al., 2002), whereas D2R *blockade* can cause an increase in ERK activation in the NAc (Bertran-Gonzalez et al., 2008; Fricks-Gleason & Marshall, 2011; Valjent et al., 2004). Taking the foregoing studies into account, we conclude from our present findings that D1Rs are the principal dopaminergic mechanism responsible for the regulation of accumbal ERK signaling by appetitive CSs.

Much evidence indicates that glutamatergic and dopaminergic signals converge at the level of the NAc. Glutamatergic NAc afferents make synaptic contacts onto the same MSN dendritic spines that receive dopaminergic input (Sesack & Pickel, 1990; Totterdell & Smith, 1989), and NMDAR and D1R are co-expressed in MSNs (Fiorentini, 2003; Hara & Pickel, 2005). MSN NMDAR activation was shown to recruit functional D1Rs to the membrane surface (Scott et al., 2002); and, conversely, D1R activation can induce NMDAR trafficking (Dunah & Standaert, 2001). Finally, NMDAR antagonism blocks D1R-induced stimulation of striatal cells (Konradi et al., 1996), and drug-induced increases in ERK signaling in the NAc requires both

NMDARs and D1Rs (Valjent et al., 2000; 2004; 2005). Together, these findings point to ERK as a convergence point between glutamatergic and dopaminergic signals in the NAc; our data suggest that this aspect of ERK also applies to the enzyme's activation by conditioned cues.

Our findings compliment numerous electrophysiological studies showing that natural reward- or drug-paired cues can change neuronal firing in the NAc (Carelli, 2002; Day et al., 2006; Roitman et al., 2005; Wan & Peoples, 2006; Yun, 2004), and that disruption of dopaminergic input to the NAc prevents cue-induced changes in NAc cell firing (Yun, 2004). It therefore is tempting to speculate that the same population of cells that exhibit increases in firing rate in response to an appetitive CS also exhibit an increase in ERK activation. Thus, pERK immunoreactivity may prove to be a tool for identification of the specific NAc cells that respond to an appetitive CS in an excitatory manner.

CREB is an important transcription factor involved in regulating many processes, including synaptic plasticity, memory, and addiction [reviewed in: (Carlezon Jr et al., 2005; Nestler, 2004)]. Phosphorylation on serine-133 activates CREB and allows for gene transcription (Gonzalez & Montminy, 1989; Impey et al., 2004; Sheng et al., 1991), [reviewed in: (Carlezon Jr et al., 2005)]. In the NAc, CREB was shown to be activated after exposure to emotionally-salient stimuli, drugs of abuse, and drug-associated cues (Kuo et al., 2007; Miller & Marshall, 2005; Muschamp et al., 2011; Tropea et al., 2008). We previously showed that CREB also is phosphorylated after exposure to appetitive cues [(Shiflett et al., 2009); this work]. Here, we demonstrate that CS-evoked CREB activation is mediated through the NMDAR-D1R/ERK signal transduction pathway. Our findings align with studies showing increased striatal CREB phosphorylation in response to application of NMDA (Das et al., 1997; Liu & Graybiel, 1998) or activation of D1Rs (Das et al., 1997; Haberny & Carr, 2005; Liu & Graybiel, 1998), and

observations that ERK inhibition prevents D1R-stimulated increases in pCREB (Haberny & Carr, 2005). Interestingly, cocaine-induced increases in CREB phosphorylation were found to be associated with increases in ERK but not CaMKIV activation in the NAc (Mattson et al., 2005). Our findings suggest that reward-predictive stimuli trigger a similar signaling pathway in NAc cells as do rewards.

Not only can CSs elicit conditioned responses, they can also modulate reward-seeking behavior. In the case of appetitive CSs, the incentive motivational effect has been implicated in relapse to drug taking (Corbit & Janak, 2007; Gipson et al., 2013; Grimm et al., 2002; LeBlanc et al., 2012). We previously showed that NAc ERK activation is critical for CS modulation of reward seeking (Shiflett et al., 2008). D1R blockade within the NAc also was shown to interfere with CS modulation of reward seeking (Dickinson et al., 2000; Lex & Hauber, 2008). Our current findings suggest that disruption of the incentive motivational effect of a CS on reward seeking by D1R blockade may be attributable to the absence of CS-evoked ERK activation in the presence of the receptor antagonist. Furthermore, our findings lead to the prediction that NMDAR blockade in the NAc interferes with CS control of reward seeking. Findings of blunted responding for cue-predicted reward in the presence of accumbal NMDAR blockade are consistent with this prediction (Bespalov & Zvartau, 1996; Giertler et al., 2003).

Conclusions: We have identified upstream mediators and a downstream effector of CS-driven ERK signaling in the NAc. CSs can cause relapse to drug use. Knowledge of the signaling pathways evoked by CSs and implicated in their behavioral consequences may prove useful in the development of strategies for curbing excessive cue control of reward seeking.

SUPPLEMENTAL INFORMATION

The following two tables were included as supplementary information for our submitted publication.

Table B.1. pERK immunoreactive cell counts[a] in the NAc of T+F rats[b] that received either APV or SCH in one NAc and vehicle in the other NAc.

pERK Cells per mm^2	Vehicle	APV	Vehicle	SCH
NAc Core	30	20	15	0
	50	15	40	5
	55	10	30	20
Mean±SEM	*45±8*	*15±3*	*28±7*	*8±6*
NAc Shell	105	25	120	35
	60	5	115	20
	95	35	95	25
Mean±SEM	*87±14*	*22±9*	*110±8*	*27±4*

[a]A counting window of 250x450µm was used in 2-3 sections per animal; counts were then averaged and converted into cells per mm^2 for each animal. [b]Three separate T+F rats were used for each drug (total n = 6). Each T+F rat received infusions of vehicle in one NAc and drug (either APV or SCH) in the other NAc.

Table B.2. pCREB immunoreactive density values[a] in the NAc of T+F rats[b] that received either APV, SCH or U0126 in one NAc and vehicle in the other NAc.

pCREB Density (arbitrary units)	Vehicle	APV	Vehicle	SCH	Vehicle	U0126
NAc Core	84	23	90	35	89	26
	66	27	89	31	73	27
	75	34	77	30	89	33
Mean±SEM	*75±5*	*28±3*	*85±4*	*32±1*	*83±5*	*28±2*
NAc Shell	128	83	98	85	104	33
	83	74	107	72	101	53
	96	53	102	87	91	48
Mean±SEM	*102±13*	*70±9*	*102±3*	*81±5*	*98±4*	*45±6*

[a]Density of positive immunoreactivity in arbitrary units, measured in a window of 250x450µm, was calculated in 2-3 sections and then averaged for each animal. [b]Three separate T+F rats were used for each drug (total n = 9). Each T+F rat received infusions of vehicle in one NAc and drug (either APV, SCH or U0126) in the other NAc.

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